

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



32
B3

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 35/14, 35/16, C12N 5/00, 5/02, 5/06		A1	(11) International Publication Number: WO 98/25634
			(43) International Publication Date: 18 June 1998 (18.06.98)
(21) International Application Number: PCT/IL97/00395			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 1 December 1997 (01.12.97)			
(30) Priority Data: 60/032,646 10 December 1996 (10.12.96) US			
(71) Applicant (for all designated States except US): HADASIT MEDICAL RESEARCH SERVICES & DEVELOPMENT COMPANY LTD. [IL/IL]; P.O. Box 12000, 91120 Jerusalem (IL).			
(72) Inventors; and (75) Inventors/Applicants (for US only): PELED, Tony [IL/IL]; 8 Karmel Street, 90805 Mevaseret Zion (IL). FIBACH, Eitan [IL/IL]; 11 Kramim Street, 90805 Mevaseret Zion (IL). RACHMILEWITZ, Eliezer, A. [IL/IL]; 33 Chachmi Street, 96428 Jerusalem (IL).			
(74) Agent: HACKMEY, Michal; A.E. Mulford, P.O. Box 544, 91004 Jerusalem (IL).			Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.
(54) Title: SERUM-DERIVED FACTOR INDUCING CELL DIFFERENTIATION AND MEDICAL USES THEREOF			
(57) Abstract <p>The present invention relates to a biologically active serum-derived composition of matter (SDF), having a low molecular weight, being electrically charged at acidic pH and having absorption at 280 nm. The molecular weight of said SDF was determined by electron spray mass spectrometry and is of 316 Da. SDF of the present invention or its complex with ceruloplasmin (CP) have several therapeutic properties. For example, SDF or its complex with CP is capable of inducing terminal cell differentiation of leukemic cells, which as a result, may lose their ability to proliferate and their ability for self cell renewal. Further, SDF or the complex with CP is capable of stimulating the proliferation of early, normal progenitor cells and inhibiting enhanced angiogenesis. In addition, SDF or its complex with CP is capable of <i>ex vivo</i> expanding normal stem and progenitor cells. The invention also relates to pharmaceutical composition comprising as active ingredient SDF or its complex and optionally further comprising pharmaceutically acceptable additives. Such pharmaceutical compositions may be for inhibiting enhanced angiogenesis, for inducing remission of tumors, for maintaining tumor remission, and for expanding hematopoietic normal stem and progenitor bone marrow transplants.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	BO	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

SERUM-DERIVED FACTOR INDUCING CELL DIFFERENTIATION AND MEDICAL USES THEREOF

FIELD OF THE INVENTION

5. The present invention relates to a biologically active serum-derived composition of matter (SDF), having a low molecular weight, being electrically charged at acidic pH and having absorption at 280 nm, to methods for the isolation thereof and to pharmaceutical compositions comprising the same.

BACKGROUND OF THE INVENTION

- 10 Normal hemopoiesis is coordinated by a variety of regulators which include glycoprotein growth factors (cytokines), such as the colony stimulating factors, as well as non-protein small molecules such as the retinoids. They regulate the survival (apoptosis), proliferation and differentiation of progenitor and precursor cells and the activation state of mature cells. Both proliferation and differentiation processes
15 are regulated by positive and negative stimuli. In acute leukemia, a block in the cell differentiation leads to a massive accumulation of proliferative, undifferentiated non-functional cells. Recently, these regulators have been used in a wide array of clinical and laboratory applications. For example, cytokines are used for treatment of patients with aplastic states such as post bone marrow (BM) transplantation,
20 radio-chemotherapy etc., as well as for *ex vivo* expansion of specific subsets of cells valuable in cell therapy (transplantation, immuno- or gene-therapy). Low molecular weight compounds, such as retinoids, have been used for induction of differentiation in leukemic cells as a therapeutic modality.

- The current approach to treatment of leukemia is based on killing the malignant
25 cells by chemo- or radiotherapy. Such treatment is not specific to the malignant cells and damages also dividing normal cells. Therefore, an alternative approach is being developed, based on inducing the undifferentiated leukemic cells to undergo

differentiation. Evidently, terminal differentiation of hemopoietic cells is associated with loss of leukemogenicity.

It has been shown that some undifferentiated myeloid leukemia cells respond to cytokines (e.g. IL-6) and undergo differentiation into mature, functional, non-dividing granulocytes or macrophages, and thereby lose their leukemogenic potential [Fibach, E., *et al.*, *Nature, New Biology* 237:276 (1972); Shabo, Y., *et al.*, *Blood* 78:2070 (1988); Fibach, E., *et al.*, *Proc. Natl. Acad. Sci. USA* 70:343-346 (1973); Inbar, M., *et al.*, *Proc. Natl. Acad. Sci. USA* 70:2577-2581 (1973); Fibach, E. & Sachs, L. J., *Cell Physiol.* 83:177-185 (1974); Hayashi, M., *et al.*, *Int. J. Cancer* 14: 40-48 (1974); Fibach, E. & Sachs, L. J., *Cell Physiol.* 86:221-230 (1975); Fibach, E. & Sachs, L. J., *Cell Physiol.* 89:259-266 (1975)].

Other differentiation inducers include dimethylsulfoxide, hexamethylene bis-acetamide, butyric acid [Collins, S.J., *et al.*, *Proc. Natl. Acad. Sci. USA* 75:2458 (1978)], derivatives of vitamins A and D3 [Breitman, T.T., *et al.*, *Proc. Natl. Acad. Sci. USA* 77:2936 (1980)] and low doses of cytotoxic drugs such as actinomycin D and cytosine arabinoside [Breitman, T.T., *et al.*, *ibid.*]. Retinoic acid has been used in the treatment of acute promyelocytic leukemia [Chomienne, C., *FASEB* 10:1025 (1996)].

Although capable of inducing some cell lines, these inducers have only rarely been found to induce terminal differentiation in cells freshly isolated from leukemic patients [Breitman *et al.*, (1980) *ibid.*].

Several publications describe some activities of the high molecular weight, copper binding protein, ceruloplasmin (CP) in malignant and aplastic states.

For example, in JP 56120622 and JP 56090015, CP is described as the active ingredient in an antitumor preparation against leukemia. JP 56120622 describes the CP as having a therapeutic activity against several mammalian tumors due to its inhibitory effect on aggravation of cancer. In addition, CP is described as being

capable of inactivating the strong oxidative super-oxide anion radicals by converting them into oxygen molecules. It is also mentioned that CP has an effect on the promotion of liver catalase biosynthesis.

JP56090015 describes a preventative and remedial drug for side reactions of anti-
5 malignant tumor agents, which contains human CP as the main ingredient.

JP 56002916 also describes CP as an anti-tumor agent. This publication is concerned with compositions for the prevention and treatment of radiation damages which contain CP as the active ingredient. Animals irradiated with γ -rays, after pre-incubation with a composition comprising CP, showed a high survival rate. The
10 preventive activity described in this publication was specifically attributed to CP.

JP 60149529 relates to the production of differentiation-inducing factors, as a result of administration of CP to mammals. In addition, medicines for leukemia in which the active ingredients are the differentiation-inducing factors produced after treating mammals with CP are described. As indicated in this publication, the differentiation
15 of leukemic cells obtained by the differentiation-inducing factors is induced via a CP stimulus. Serum obtained from rabbits which repeatedly received CP was capable of inducing differentiation of M1 cells into macrophages. However, CP by itself was incapable of inducing the differentiation. There is no indication as to the identity or nature of the substance obtained by CP stimulus, which causes the
20 induction of differentiation.

The use of CP has been described also for the preparation of other pharmaceutical compositions. For example, GB 1,304,697 describes pharmaceutical compositions comprising CP for use, in particular, against inflammation.

Further, clinical trials have shown that CP may be helpful in therapy of aplastic
25 anemia [Shimizu, M., Transfusion 19(6):742-8 (1979); Arimori, S., Jap. J. Clin. Exper. Med. 43:1897 (1966)].

Contrary to the earlier reports, the present invention reveals that the activities previously ascribed to CP, should be attributed to a small molecular weight composition of matter (SDF), which in serum is preferentially associated with CP (SDF-CP complex). Regardless of the nature of association between SDF and CP, it is clear that CP derived from adult serum is a rich source of the factor. Other sources for SDF are urine and fetal serum. Due to its large molecular weight, intact CP cannot be present in the urine. Thus, it is assumed that the factor present in urine is not associated with the CP molecule, or at least not with the intact molecule.

As will be shown hereafter, SDF, as well as its complex with CP, which are the subject of the present invention, may have many therapeutical uses.

SUMMARY OF THE INVENTION

The present invention relates to a biologically active serum-derived composition of matter (SDF), having a low molecular weight, being electrically charged at acidic pH and having absorption at 280 nm. The molecular weight of SDF, as determined by electron spray is 316.

In a second aspect, the invention relates to a method for the isolation from plasma and purification of a low molecular weight composition of matter, comprising the steps of (a) transferring plasma through an affinity column to obtain an electrophoretically homogeneous fraction being the SDF-CP complex, which may be optionally concentrated by transferring through an anion exchange column; (b) isolating the SDF from the SDF-CP complex by either (i) transferring the fraction obtained in step (a) through RP-HPLC "Resource"™ column, elution buffer A consisting of 0.05-0.1% trifluoroacetic acid (TFA) in water (pH 2.5), elution buffer B consisting of acetonitrile, the fractions eluted at acetonitrile concentration of 0-2% and 13-17% being collected and combined, or (ii) extracting the fraction obtained in step (a) with an acidified solvent, wherein the active fraction is recovered from the organic phase; (c) purifying the fraction obtained in (b) by

RP-HPLC chromatography separation using a C-18 column, wherein in a first optional separation step elution buffer A, consisting of 0.1% TFA in water (pH 2.5), and elution buffer B, consisting of 0.1% TFA in acetonitrile, are employed, the fraction eluted at acetonitrile concentration of 0-2% being collected, and in the subsequent separation step, elution buffer A, consisting of 0.1% triethylamine in water and adjusted to pH 7.0, and elution buffer B, consisting of acetonitrile, are employed, the fraction eluted at acetonitrile concentration of 9-11% being collected.

Also within the scope of the invention is a biologically active complex comprising ceruloplasmin and said biologically active composition of matter (SDF).

In a second aspect, the invention relates to pharmaceutical composition comprising as active ingredient the SDF of the invention or its complex with CP, and optionally further comprising pharmaceutically acceptable additives.

Such pharmaceutical compositions may be used for treatment of patients with aplastic marrow, for inducing or maintaining remission of tumors, for expanding hematopoietic normal stem and progenitor cells for bone marrow transplants or for inhibiting enhanced angiogenesis.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Affinity purification of the SDF-CP complex from plasma

The fraction obtained following precipitation of plasma with 30-60% ammonium sulfate was equilibrated in 10 mM tris buffer, pH 7.4, conductivity - 5 mS, and separated on tentacle agarose gel (200 ml bed volume equilibrated in the same buffer). Stepwise elution was performed with 0.1, 0.2, 0.3, 0.5, 1.0 M NaCl in tris buffer and the eluates were analyzed for activity. SDF activity was found to be eluted with 0.5 M NaCl (peak 6).

Fig. 2: Separation of SDF from its high MW complex with CP on RP-HPLC 'Resource' column

The fraction was separated from its complex and is depicted by the absorbance at 220 nm (A_{220}) as function of time ($T(\text{min.})$). Buffer A - 0.1% TFA in H_2O (pH 2.5), buffer B - 0.1% TFA in acetonitrile. The gradient is indicated as the acetonitrile percentage in the eluting buffer (ACN(%)), Flow - 140 ml/min. SDF fraction is eluted in correlation with the void and after 5-6 min (a).

Fig. 3: RP-HPLC separation of SDF from co-eluted contaminants at pH 2.5

Active fractions derived from the resource separation of Figure 1 were further separated on a C18 (Vydac 2.1x280) RP-HPLC column, which is depicted by the absorbance at 220 nm (A_{220}) as a function of time ($T(\text{min.})$). Buffer A - 0.1% TFA in H_2O (pH 2.5), buffer B - 0.1% TFA in acetonitrile. The gradient is presented as a function of acetonitrile percentage in the eluting buffer (ACN(%)), Flow -140 ml/min. SDF activity was eluted after 5-6 min.

Fig. 4: RP-HPLC separation of SDF from co-eluted contaminants-at pH 7

'Resource' derived fraction at 5-6 min was further separated on C-18 (Vydac 2.1x280) RP-HPLC at pH 7 and is depicted by the absorbance (A_{220}) as a function of time ($T(\text{min.})$). Buffer A: 0.1% triethylamine in H_2O adjusted to pH 7.0 with TFA. Buffer B: acetonitrile. Flow: 140 ml/min. The gradient is illustrated as the acetonitrile percentage in the eluting buffer (ACN(%)). The activity was recovered at 11.26 min. in correlation with a single symmetric peak.

Fig. 5: Purification of SDF on RP-HPLC

Active fractions, pooled from the separation on RP-HPLC at pH 7, were re-chromatographed on C-18 at pH 2.5 as depicted in the figure by the absorbance (A_{220}) as function of time ($T(\text{min.})$). The gradient is indicated

by the acetonitrile percentage (ACN%) in the eluting buffer. The activity was recovered from the single peak eluted at 6.73 min.

Fig. 6: UV spectrum of purified SDF

Purified SDF obtained as described in the following Examples was analysed by UV spectra. The figure illustrates the absorbance (A) as a function of the wavelength (W) in nm.

Fig. 7: Mass Spectrum of SDF

Purified SDF obtained as described in the following Examples was analysed by mass spectrometry (electron spray).

Fig. 8: Fragmentation and analysis of the 316 MW fraction by Mass Spectrometry

A: Fragmentation at cone voltage - 30 V.

B: Fragmentation at cone voltage - 45 V.

C: Fragmentation at cone voltage - 60 V.

Fig. 9: Effect of SDF on myeloid and erythroid colony growth

A: Light density peripheral blood (PB) cells were cultured in liquid culture (phase I, as described in the Examples) supplemented with none (C, for control), 5637 CM (10% v/v, (CM)), SDF and SDF-CP complex (SDF-CP) at different dilutions. After 5 days the cells were harvested, washed and cloned in semi-solid medium supplemented with Epo. Colonies were scored on day 14 as illustrated in the figure by the number of erythroid colonies per plate (No. e.c/p).

B: Light density PB cells were cultured in liquid culture (phase I) supplemented with 5637 CM (10% v/v, (CM)) or SDF (1:200, (SDF)). After 5 days the cells were harvested, washed and cloned in semi-solid medium supplemented with 5637 CM for myeloid colonies, or with Epo for erythroid colonies. Colonies were scored on day 14 and is illustrated in the figure by the number of colonies per plate (No. C/P) both for erythroid (e) and for myeloid (m) cells.

Fig. 10: Effect of SDF on CD34⁺ cells

Light density PB cells were cultured in liquid culture (phase I) supplemented with 5637 CM (10% v/v(CM)), stem cell factor (SCF) or SDF (SDF). CD34⁺ cells were enumerated by flow cytometry (CD34⁺(%)). At the initiation of the culture the percentage of CD34⁺ cells (CD34⁺ (%))ranged from 0.1 to 0.5. Three independent experiments are presented (Exp. 1 to Exp. 3). The percent of CD34⁺ cells was determined in experiment (1) on day 6, in experiment (2) on day 3 and in experiment (3) on day 7.

Fig. 11: Effect of SDF on dendritic colony growth

Light density PB cells were cultured in liquid culture (phase I) supplemented with 5637 CM (10% v/v, (CM)), SDF or SDF + 5637 CM (SDF+CM). After 3-4 days, the cells were harvested, washed and cloned in semi-solid medium. Dendritic colonies (D.C %) were scored on day 14.

Fig. 12: Effect of SDF on erythroid progenitors derived from pure red cell aplasia

Low density PB cells derived from a patient with pure red cell aplasia were cultured in liquid culture (phase I) supplemented with 5637 CM (10% v/v, (CM)) or SDF + 5637 CM (SDF+CM) with the different dilution of SDF indicated in the figure. After 4 days the cells were harvested, washed and cloned in semi-solid medium supplemented with Epo. Colonies were scored on day 14, and is depicted in the figure by the number of erythroid colonies per plate (No. e.c/p).

Fig. 13: Effect of SDF on colony formation by leukemic and normal progenitors

Leukemic HL-60 cells (HL-60) and normal BM (NBM) progenitors were cloned in agarose cultures stimulated by GM-CSF (100 U/ml) and several dilutions of SDF (SDF(Dil.)). Colonies were scored on day 10. The results are illustrated as colony number as a function of the different SDF

dilutions), for HL-60 cells, two sets of experiments (Exp. 1 and Exp. 2) were conducted.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a biologically active serum-derived composition of matter (SDF), having low molecular weight, being electrically charged at acidic pH, and having absorption at 280 nm. The molecular weight of SDF being 316, as determined by electron spray mass spectrometry.

More specifically, SDF is comprised of an aromatic moiety, and carries a double charge, as indicated by the fragmentation thereof on electron spray mass spectrometry.

The invention also relates to a method for the isolation and purification from plasma, of a low molecular weight composition of matter, comprising the steps of: (a) transferring plasma through an affinity column to give an electrophoretically homogeneous fraction being the SDF-CP complex, which may optionally be concentrated by transferring through an anion exchange column and collecting the bound fraction, having an absorption at 280 nm; (b) isolating the SDF from its complex with the high MW CP protein by either (i) transferring the fraction obtained in step (a) through RP-HPLC "Resource"™ column, elution buffer A, consisting of 0.05-0.1% TFA in water (pH 2.5), and elution buffer B, consisting of acetonitrile, are employed, the active fractions eluted at acetonitrile concentration of 0-2% and 13-17% being collected and combined, said CP fraction being eluted at acetonitrile concentration of about 40% and being devoid of activity; or (ii) extracting the fraction obtained in step (a) with an acidified solvent, wherein the active fraction is recovered from the organic phase whereas CP is recovered from the aqueous phase, in an inactive form. As will be shown hereafter, the two fractions obtained in step (b) were active. The difference in the retention time may be attributed to the state of ionization of the molecule and/or its association with some

impurities, probably of small peptides (Fig 2). The affinity chromatography column employed in step (a) is preferably a tentacle-agarose gel, derived using a reaction of Sepharose CL-6B or Sepharose 4B with chloroethylamine [Calabrese, L., Biochem. Int. 16:199-208 (1988)].

5 Another purification step is required in order to isolate the SDF from its impurities. The method of the invention thus further comprises (c) purifying the active fraction obtained in step (b) by RP-HPLC chromatography separation, using a C 18 column, wherein in a first optional separation step, elution buffer A, consisting of 0.1% TFA in water (pH 2.5), and elution buffer B, consisting of 0.1% TFA in acetonitrile, are
10 employed, the fraction eluted at acetonitrile concentration of 0-2% being collected (void). This purification step at acidic pH (2.5) results in a partial disassociation of SDF from said impurities. The active fraction is eluted in correlation with the void volume, while most of the impurities are eluted with the gradient. The semi-purified SDF (in case the optional purification at acidic pH is employed) is then subjected to
15 a subsequent separation step, by transferring the same through the same column, employing elution buffer A, consisting of 0.1% triethylamine in water, adjusted to pH 7.0, and elution buffer B, consisting of acetonitrile. The fraction appearing as a single, symmetrical peak, is eluted with acetonitrile concentration of 9-11% and collected. Optionally, the fraction obtained from step (b) is directly separated on the
20 second separation step of (c).

A five-step purification procedure may be employed as an alternative to step (a). This five-step procedure comprises the steps of ammonium sulfate precipitation, anion exchange chromatography (DEAE), cation exchange chromatography (S-Sepharose), dye-ligand (Affigel blue) chromatography and hydrophobic
25 chromatography (TSK-Phenil). Following the last purification step, the purified fraction may be further separated on an SDS-gel.

According to the invention the plasma from which SDF is obtained is human plasma. However, the inventors have found that an active fraction is also present in

-11-

non-human plasma, in human urine and in bovine fetal serum. Since the full CP protein is too large to be present in the urine, it is assumed that the active fraction present therein is the SDF itself or in association with part of the CP protein or with other small peptides. In view of the above, SDF may be isolated and purified from human or non-human adult or fetal serum or urine, by any suitable method.

The invention also relates to a biologically active complex comprising CP and SDF.

Obviously, any biochemically pure SDF obtained by the method according to the invention are also within the scope of the present invention. The complex of SDF with CP can be obtained, for example, from step (v) of the five-step purification (Example 1B) procedure or after the one step affinity purification procedure of the method of the invention.

As shown in the description hereafter, the present inventors have found that normal serum, which sustains the growth and viability of cells in culture, contains a small molecular weight, natural product that exhibits dual activity on hematopoietic cells: on the one hand, it is extremely potent in stimulating development of a variety of normal blood cells, and, on the other hand, it inhibits leukemic cell growth by inducing terminal differentiation. Since this natural product was purified from serum it was termed "serum-derived factor" (SDF).

Effect of SDF on Normal Hemopoiesis

Use of hemopoietic growth factors in transplantation

Transplantation of hemopoietic cells, originally obtained from either autologous or allogeneic sources, has become the treatment of choice for a variety of inherited or malignant diseases. Recently, more defined populations, enriched for pluripotent hemopoietic stem cells (CD34⁺ cells) have been used in such treatments [Van Epfs, D.E., *et al.*, Blood Cells 20:411 (1994)]. In addition to bone marrow, stem cells could be derived from other sources, such as peripheral blood and neonatal umbilical cord blood [Emerson, S.G., Blood 87:3082 (1996)]. Compared to

autologous bone marrow transplantation, transplantation with peripheral blood cells shortens the period of pancytopenia and reduces the risks of infection and bleeding [Brugger, W., *et al.*, N. Engl. J. Med. 333:283 (1995); Williams, S.F., *et al.*, Blood 87:1687 (1996); Zimmerman, R.M., *et al.*, J. Hematotherapy 5:247 (1996)]. An additional advantage of using peripheral blood for transplantation is its accessibility. However, the limiting factor in peripheral blood transplantation is the low number of circulating CD34⁺ cells. Therefore, in order to obtain enough cells for transplantation, peripheral blood derived stem cells are "harvested" by repeated leukophoreses, following their mobilization from the marrow into the circulation after treatment with colony stimulating factors and chemotherapy [Brugger *et al.* (1995), *ibid.*; Williams *et al.* (1996) *ibid.*].

Preliminary attempts have been made to enrich the CD34⁺ population by *ex vivo* expansion in tissue culture containing mixtures of growth factors [Koller, M.R., *et al.*, Blood 82:378 (1993); Lebkowski, J.S., *et al.*, Blood Cells 20:404 (1994)]. Such expansion of functional stem cells from a small number of CD34⁺ cells may have the following advantages:

- It may reduce the volume of blood required for reconstitution of an adult hemopoietic system and may obviate the need for mobilization and leukophoresis [Brugger *et al.* (1995) *ibid.*].
- It may enable storage of small number of peripheral blood, bone marrow or cord blood CD34⁺ cells for potential future use of the *ex vivo* expanded population.
- In the case of autologous transplantation in patients with malignancies, decreasing the total volume of blood used and selecting CD34⁺ cells may reduce the load of tumor cells in the final transplant. Such contaminating tumor cells in autologous infusion can contribute to the recurrence of the disease [Brugger *et al.* (1995) *ibid.*].
- The cultures may provide a significant depletion of T-lymphocytes, which may be useful in the allogeneic transplant setting for reducing graft-versus-host disease.

Clinical studies have indicated that transplantation of *ex vivo* expanded cells derived from a small number of peripheral blood CD34⁺ cells can restore hemopoiesis in patients treated with high doses of chemotherapeutical agents. Nevertheless, the up to date results do not allow for firm conclusions about the long term *in vivo* hemopoietic capabilities of such cultured cells [Brugger *et al.* (1995) *ibid.*; Williams *et al.* (1996) *ibid.*].

For successful transplantation, shortening the duration of the cytopenic phase, as well as long-term engraftment, is crucial. Inclusion of intermediate and late progenitor cells in the transplant could accelerate the production of donor-derived mature cells and shorten the cytopenic phase. It is important that *ex vivo* expanded cells will include, in addition to stem cells, more differentiated progenitors in order to optimize short-term recovery and long term restoration of hemopoiesis. For this purpose, expansion of intermediate and late progenitor cells, especially those committed to the neutrophilic and megakaryocytic lineages, concomitant with expansion of stem cells, is required [Sandstrom, C.E., *et al.*, Blood 6:958 (1995)].

Regarding the autologous transplantation in patients with malignancies, it should be noted that different growth factors, e.g. G-CSF and GM-CSF, are currently used in BM transplantation. They have been shown to shorten the time of neutrophil recovery after transplantation (and chemotherapy), by stimulating myeloid progenitors. However, since myeloid leukemic cells have receptors for these factors, the proliferation of residual malignant cells is also stimulated. Since SDF itself, or its complex with CP, alone, or in combination with GM-CSF, potentiates the proliferation of normal progenitors, but inhibit "spontaneous" and GM-CSF stimulated proliferation of myeloid leukemic cells (Examples 2 to 6), it may have a dual effect: eradication of leukemic cells concomitantly with stimulation of the normal ones.

As exemplified hereafter, leukemic cells, treated with SDF lose their proliferation ability which indicates the cells have lost their leukemogenic potential. Therefore, it is believed that SDF may be of great therapeutical value.

Further, SDF or its complex with CP, is capable of stimulating proliferation of early, normal progenitor cells. Therefore SDF or its complex with CP may be used for *ex vivo* expansion of normal hematopoietic cells such as stem cells (CD34⁺) and myeloid and erythroid-committed progenitors as well as antigen-presenting dendritic cells, for BM transplantation treatment, or be utilized, for *ex-vivo* expansion of specific sub-populations that should be valuable in cell therapy (transplantation, and immuno- or gene therapy). In addition SDF or its complex with CP can be applied *in vivo*, where they may support the recovery of the hemopoietic tissue in aplastic states such as in aplastic anemia or following radio/chemotherapy.

In addition, although effective in inducing differentiation and inhibiting proliferation of leukemic cells, it was found by the inventors that neither SDF nor its complex with CP inhibit normal myeloid or erythroid development. Moreover, SDF or its complex with CP, alone or in combination with other growth or proliferation factors, was found to stimulate the proliferation of normal early progenitor cells. For example:

- (a) *In vitro* stimulation of early hemopoietic stem and committed progenitor cells.
- SDF was found to stimulate the amplification of early stem (CD34⁺) cells derived from PB or BM and therefore may be applied for *ex-vivo* expansion of pluripotent stem cells as well as lineage (granulocytic, erythroid and megakaryocytic) committed progenitor cells. In combination with late growth factors (added to phase 2, described hereafter in the Examples) SDF increases the number of myeloid and erythroid colony forming cells. Such cultures are important in transplantation of CD34⁺ enriched populations derived from (immobilized) PB and neonatal cord blood and in gene therapy.

-15-

- (b) *In vivo* stimulation of early stem and committed progenitor cells. SDF was found to stimulate *in vitro* proliferation of progenitor cells derived from the PB of patients with pure red cell aplasia. These results suggest that SDF or its complex with CP be administered, for recovery of normal hemopoiesis, to patients with aplastic states, such as aplastic anemia, Fanconi's anemia, myelodysplastic syndrome or following myeloablative radio/chemotherapy and BM transplantation.
- (c) *Ex vivo* expansion of specific populations of subsets of lympho-hematopoietic cells with therapeutic potential such as the antigen presenting dendritic cells. Dendritic cells are "professional", immunostimulatory, antigen-presenting cells. Various studies have suggested the potential use of dendritic cells in immunotherapy. This modality involves infusion of dendritic cells pulsed *in vitro* with tumor antigens as therapeutic vaccines, as well as using dendritic cells for priming tumor antigen specific T cells *in vitro* for use in adoptive T cell therapy [Bernhard, H., *et al.*, Cancer Res. 55:1099 (1995); Protti, M.P., *et al.*, Cancer Res. 56:1210, (1996)]. According to the literature, the best "cocktail" for growing such cells is a mixture of cytokines (GM-CSF, SCF, IL-4, TNF α). When BM cells were cloned in the presence of such a cocktail, 40% of the total number of the developed colonies, contained dendritic cells [Moore, M.A., *et al.*, J. Exp. Med. 182:1111 (1995)]. In order to obtain such colonies from PB progenitors, the cultured population should be enriched for CD34⁺ cells. SDF or its complex with CP induce commitment/expansion of PB CFU-dendritic. Using SDF or its complex with CP (without TNF or SCF), up to 80% dendritic colonies were obtained from non-enriched PB mononuclear cells.
- As will be shown in Example 5, SDF has a potent inhibitory activity on the proliferation of endothelial cells from bovine aorta.

-16-

As mentioned above, in addition to BM transplantation, *ex vivo* expansion of hematopoietic stem cells using SDF or its complex with CP, may be used in gene therapy.

Effect of SDF on Leukemic hemopoiesis

5 In addition, SDF or its complex with CP, whether obtained by the method of the invention or by any other suitable method, or synthesized by any suitable procedure, possess several therapeutical activities such as inducing differentiation and inhibiting proliferation of both human and murine established leukemic cell lines and of freshly explanted cells from acute and chronic human myeloid leukemias. In
10 addition, SDF itself or its complex with CP are capable of inducing terminal cell differentiation of leukemic cells. Blast cells lose their leukemic phenotype and turn into functional, non-dividing macrophages. Further, either as a result of said terminal cell differentiation or independent therefrom, said leukemic cells, in the presence of SDF or its complex with CP, lose their ability to proliferate and their
15 ability for self cell renewal. The effect of SDF, or its complex with CP, on leukemic cells makes it potentially useful in the treatment of myeloid leukemias in three clinical settings: (a) for induction of remission, optionally, in combination with other hemopoietic factors or low-dose chemotherapy, using "differentiation-inducing therapy" as the main modality; (b) for maintenance of remission state of
20 tumors and (c) in autologous transplantation, for either *in vitro* or *in vivo* purging of residual leukemic cells.

In a different aspect, SDF or its complex with CP, may be utilized for regulating the proliferation and differentiation of hemopoietic cells, by modulating nuclear transcription factors.

25 Modulating the level of expression of specific genes is a prerequisite for controlling cellular growth and differentiation. Gene expression is controlled by sequence-specific DNA binding proteins (transcription factors) which in certain cases are targets for signal transduction from cell surface receptors. The importance of this

-17-

process for growth control is emphasized by the finding that several proto-oncogenes, including *c-Myc*, *c-Myb*, *c-Fos*, *c-Jun*, etc. encode sequence-specific transcription factors [Xanthoudakis, S., EMBO J 11:3323 (1992); Ammendola, R., Eur. J. Biochem. 225:483 (1994)]. Although the activity of these factors can be modulated by phosphorylation, recent evidence has emerged for an additional form of regulation of DNA binding activity which is mediated by changes in reduction-oxidation (redox) status. It is suggested that redox status could provide a general mechanism for post-translational control of transcription factors in an analogous fashion to phosphorylation [Xanthoudakis (1992) *ibid.*; Ammendola (1994) *ibid.*].

For example, the binding of Fos-Jun hetero-dimers and Jun-Jun homo-dimers to DNA requires that these proteins be in a reduced state. This form of redox regulation may be widespread because the DNA binding activities of several other transcription factors, including *Myb*, *Rel*, and NF-kB are sensitive to changes in their oxidation state in a similar manner.

Recent disclosures suggest the contribution of small redox-potential molecules such as glutathion or large proteins such as thioredoxin [Walker, L.J., Mol. Cell. Biol. 13:5370 (1993)] to the regulation of DNA binding ability of several nuclear transcription factors like Sp-1. In human fibroblast cultures it was shown that small molecules with redox potential like pyrroloquinoline quinone (PQQ) stimulate proliferation [Naito, Y., Life Sciences 52:1909 (1993)]. The potency of PQQ was shown to be comparable to that of epidermal growth factor and is much higher than that of fibroblast growth factor or insulin growth factor. Pyrrolidine derivatives of dithiocarbamates trigger myeloid differentiation through AP-1 regulation [Aragones, J., J. Biol. Chem. 271:10924 (1996)]. Therefore, it may be concluded that small molecules with redox potential activity could modulate cell proliferation and differentiation via the regulation of transcription factors activity.

It was found by the inventors, that PQQ at high concentrations can induce differentiation of leukemic cells and stimulate proliferation of normal cells

(Example 6). Nevertheless, in view of the high concentrations required, and compared to SDF, the efficiency of PQQ is low. Nevertheless, since SDF is a low molecular weight composition of matter, believed to be carrying a double negative charge, and in view of its potency in inducing differentiation, it is anticipated that, similarly to PQQ, SDF possesses a redox potential activity and consequently might modulates cell proliferation and differentiation via the regulation of transcription factors activity.

Effect of SDF on Angiogenesis

In a different aspect, SDF was found to have a potent inhibitory activity on endothelial cell proliferation, and therefore it might be applicable for inhibiting angiogenesis. In certain pathological conditions angiogenesis is dramatically enhanced and is no longer self-limited. Pathological angiogenesis is seen during the development of many diseases, for example rheumatoid arthritis, psoriasis, retrolental fibroplasia, diabetic retinopathy and hemangiomas, during the rejection of organ transplants and most importantly in solid tumor malignancies. Well vascularized tumors expand both locally and by metastasis, while avascular tumors do not grow beyond a diameter of 1-2 mm. It has been suggested that this is the results of lack of balance between angiogenic stimulators and inhibitors [Folkman, J., New Engl. J. Med. 285:1182-1186 (1971); Folkman, J., J. Natl. Cancer Inst. 82: 4-6 (1989)].

In another aspect, pharmaceutical composition comprising as active ingredient SDF or its complex with CP and optionally further comprising pharmaceutically acceptable additives are within the scope of the invention. Such pharmaceutical compositions may be used for inhibiting enhanced angiogenesis in diseases where uncontrolled angiogenesis is associated with the pathological manifestations.

Other pharmaceutical composition of the invention may be for inducing remission of tumors comprising as active ingredient the SDF of the invention, and optionally further comprising pharmaceutically acceptable additives.

-19-

Alternatively, the pharmaceutical composition of the invention can be used for maintaining tumor remission state comprising as active ingredient the SDF of the invention, and optionally further comprising pharmaceutically acceptable additives.

Further, pharmaceutical composition for expanding hematopoietic normal stem and progenitor bone marrow transplants comprising as active ingredient SDF, and optionally further comprising pharmaceutically acceptable additives are also within the scope of the invention.

The magnitude of therapeutic dose of the SDF on the invention will of course vary with the group of patients (age, sex etc.), the nature of the condition to be treated and with the route administration and will be determined by the attending physician.

The pharmaceutical composition of the invention can be prepared in dosage unit forms. The dosage forms may also include sustained release devices. The compositions may be prepared by any of the methods well-known in the art of pharmacy.

In the pharmaceutical compositions of the pharmaceutically acceptable additives may be any pharmaceutical acceptable carrier, excipient or stabilizer, and optionally other therapeutic constituents. Naturally, the acceptable carriers, excipients or stabilizers are non-toxic to recipients at the dosages and concentrations employed.

Finally, within the scope of the invention, is the use of SDF or its complex with CP, in the preparation of a variety of pharmaceutical compositions.

EXAMPLES

Example 1 - Separation and purification of SDF

A: One step affinity purification of the SDF-high MW complex

In order to separate the CP from the plasma, a human plasma was transferred through an affinity column. The affinity chromatography procedure is based in the tentacle-agarose gel procedure [Robert, V.S., Biochemistry International 27:281-289

(1992)], which preferentially binds the CP protein. The gel was derived by reacting Sepharose CL-6B or Sepharose 4B with chloroethylamine [Robert (1992) *ibid.*].

Optionally, prior to transferring the plasma through an affinity column, the plasma may be precipitated by ammonium sulfate, at a cut-off of 30-60%, equilibrated in 10mM tris buffer pH 7.4, conductivity 5 ms and then separated on a tentacle-agarose gel as described above. Elution was performed stepwise with: 0.1, 0.2, 0.3, 0.5, 1.0 M NaCl in tris buffer (Figure 1).

In order to concentrate the electrophoretically homogeneous CP fraction obtained from the affinity chromatography, the fraction was transferred through an anion exchange column (DEAE, or QAE Sephadex columns). The column and the fraction were equilibrated with 300 mM NaCl in tris buffer. The bound fraction was eluted with 300 mM NaCl in tris buffer.

B: five step purification of the SDF-high MW complex

(i) *Ammonium sulfate precipitation* - Thirty liters of human plasma were centrifuged. To the clear supernatant solid ammonium sulfate was added to a final concentration of 60% and then stirred for 4 hr at room temperature. The slurry mixture obtained was centrifuged, the precipitate discharged. An additional amount of solid ammonium sulfate was added to the supernatant up to 85% saturation. After 24 hrs of stirring, the slurry mixture was centrifuged and the precipitate dissolved in 6 L of tris buffer 10 mM (pH 7.5), 50 mM NaCl, and diafiltered against the same buffer, using 3K cut off membrane. The diafiltration was completed when conductivity reached 5 mS and the volume of the solution reduced to 4L. The solution obtained was clear, no pellet was seen upon centrifugation at 14000xg for 10 min., 2.4x10 mg of protein were recovered.

(ii) *Anion exchange chromatography* - The fraction obtained from step (i) was equilibrated with tris buffer 20 mM (pH 7.4), 120 mM NaCl (conductivity 120 mS), and pumped on a DEAE column (Pharmacia LKB Biotechnology AB,

-21-

Uppsala Sweden, bed volume 1 liter (11.2dx10h)), previously equilibrated with the same buffer at flow 2.5 L/hr. The column washed with the loading buffer until the effluent O.D. at 280 nm returned to the base-line. The elution buffer employed was a linear salt gradient from 120 mM NaCl to 500 mM NaCl in tris buffer. The activity was eluted with 300 mM NaCl.

The DEAE-derived fractions were diluted v/v with tris buffer 25 mM (pH 7.4), 100 mM NaCl. n-Butanol was added dropwise while stirring until an upper butanol phase was obtained. After centrifugation the two phases (aqueous and butanolic) were collected separately. The butanolic phase was evaporated and redissolved in ethanol or in the tris buffer and assayed for activity. The aqueous phase was further separated on a DEAE column. The bound protein was step eluted with tris buffer (pH 7.4), 1M NaCl. The activity was recovered from the bound fraction.

(iii) *Cation exchange chromatography* - The aqueous fraction obtained from the butanol extraction and DEAE step elution was further separated on a S-Sepharose column (Pharmacia, 800 ml bed volume). Elution buffer A consisting of 20 mM acetate buffer (pH 5.0), 40 mM NaCl (conductivity 4 mS), elution buffer B consisting of 20 mM acetate buffer (pH 5.0), 0.6 mM NaCl. The column, equilibrated with buffer A was loaded with fraction, adjusted to the conditions of buffer A. Elution gradient employed was a linear salt gradient, starting from 100% buffer A, to 100% buffer B. The activity was eluted with 300 mM NaCl.

(iv) *Dye-ligand (Affigel blue) chromatography* - The pooled active fraction obtained from the S-Sepharose chromatography was separated on an affinity Gel Blue column (50-100mesh, Bio-Rad, Cat. No. 153-7301, column bed volume 40 ml). Elution buffer A consisting of 50 mM tris buffer (pH 7.4), 24 mM KCl and 2 mM ZnCl (conductivity 5.5 mS), elution buffer B consisting of 50 mM tris buffer (pH 7.4), 24 mM KCl and buffer C consisting of 50 mM tris buffer (pH

-22-

7.4), 1 M KCl, were employed. The column previously equilibrated with buffer A was loaded with the active fraction which was adjusted to the conditions of buffer A and pumped at flow 3 ml/min. The protein was eluted with a step elution employing 100% buffer.

- 5 (v) *Hydrophobic chromatography* - Pooled active fraction obtained from the Affigel blue was further separated on a TSK-phenyl column (Pharmacia, bed volume 15 ml). Elution buffer A consisting of 50 mM tris buffer (pH 7.4), 20% ammonium sulfate, buffer B consisting of 50 mM tris buffer (pH 7.4) and buffer C consisting of 20% ethanol in 50 mM tris buffer (pH 7.4). The column
10 previously equilibrated with buffer A was loaded with the active fraction, adjusted to the conditions of buffer A. Negative salt gradient (ammonium sulfate) was employed, from 100% buffer A to 100% buffer B, followed by a continuous step elution with buffer C. The activity was eluted with 5-10% ammonium sulfate.

- 15 The five-step procedure is summarized in Table 1.

In either procedure (A or B), an electrophoretically homogeneous fraction of SDF in its complex with CP was obtained (97KD MW), (data not shown). Seventy percent of the SDF activity was recovered from the 97KD band whereas 30% of the activity was recovered from a fraction in correlation with the front of the gel.

-23-

Table 1

Purification of SDF-complex - Summary

Purification steps	Protein (mg)	Units $\times 10^5$	Specific activity	Purification (fold)	Recovery (%)
Human plasma (30 liters)	2.4×10^6	-	-	-	-
Ammonium sulfate + Ion Exchange chromatography	4.76×10^3	3	63	600	100
N-butanol extraction + DEAE (step elution)	1.3×10^3	2.8	215	2220	93
Dialysis (pH 5)	700	2.3	329	4100	82
S-Sepharose	70	1.6	2286	41000	70
Affi-Gel Blue	10	0.8	8000	287000	50
TSK Phenyl	5	0.7	14000	574000	87.5

Total purification - 480,000 fold

SDF recovery - 23%

- 5 Following the last purification step, the purified fraction may be separated on an SDS-gel: 70% of the activity was recovered from a protein band that correlates with MW of 97KD while 30% of the activity was recovered from a low MW fraction at the front of the gel. The 97KD band was sequenced and found to correspond to the copper binding protein- ceruloplasmin. The low MW fraction was also sequenced
- 10 and analyzed by mass spectrum and found to contain several peptides of 1-2 KD MW. In order to separate the SDF molecule from the high MW complex obtained in step (v), the complex was subjected to purification by steps (b) and (c) described above.

SDF Isolation from its high MW complex with CP

The SDF fraction can be isolated from the complex in two alternative procedures:

A: *RP-HPLC "Resource"™ chromatography* - The active fraction was separated from 9 mg purified complex on a RP-HPLC column (3 ml, Pharmacia) comprised of polystyrene/divinyl benzene beads. (Fig. 2). Buffer A consisting of 0.1-0.05% TFA in H₂O, buffer B consisting of acetonitrile, flow 3 ml/min, absorption at A₂₂₀. Two SDF peaks at 0-2% and 13-17% acetonitrile were detected and collected.

B: *solvent extraction* - The active fraction was separated from its complex with CP by solvent extraction with an acidified solvent. A mixture of SDF-CP, 1 M HCl and butanol (1:4:10) was shaken and then centrifuged (at about 2000xrpm) for 5 min. The aqueous and organic phases were collected separately. The SDF activity was recovered in the organic phase while the CP fraction was present in the aqueous phase. Methanol, acetonitrile or chloroform may also be used as the solvent.

SDF purification

RP-HPLC chromatography - Purification of SDF by RP-HPLC separation procedure, employing a C-18 column (Vydac 2.1x280 mm).

In a first purification step an elution buffer having a pH of 2.5 was employed. buffer A - 0.1 TFA in H₂O, and buffer B - 0.1% TFA in acetonitrile. The SDF fraction was eluted with about 0-2% acetonitrile, after 5-6 min (Fig. 3), which correlated with the void volume.

In a second purification step, buffer A consisting of 1% triethylamine in water, adjusted to pH of 7.0 and buffer B consisting of acetonitrile were employed. The SDF fraction was eluted with about 9-11% acetonitrile, after 11-12 min (Fig. 4).

The fractions eluted at 11.26 min, from the second RP-HPLC separation step (Fig. 4), were pooled and rechromatographed with the same conditions as in the first RP-HPLC separation step, only to obtain a single, symmetrical peak at 5-6 min. The

purpose of the rechromatography step, is to show that the fraction obtained after the second separation is a single homogenous peak (Fig. 5).

Example 2 - Characterization of SDF

Ultraviolet absorption

5 Ultraviolet absorption spectrum of the active peak eluted from the C-18 column (Figs. 4-5) suggests the presence of a dominant aromatic compound as indicated by the absorbance at 280 nm (Fig 6). This fraction was further subjected to amino acid analysis and sequencing, the results of which were both negative. Therefore, it was concluded that the SDF, although purified up to this step in association with
10 peptides, the active compound is most likely not a peptide.

Mass spectroscopy

Mass spectroscopy (electron spray) analysis of the active fraction (Fig. 7) indicates the presence of a single compound with a molecular weight of 316 (317-1). Fragmentation of the molecule at cone voltage 30, 45 and 60 V is shown in Fig 8 (a-
15 c). The 159 molecular mass fragment may represent half the mass of the compound. Such a half mass fragmentation is characteristic to a double charged molecule ($M/2e$). In addition, double charged ions are mostly found in spectra of highly unsaturated compounds (e.g. aromatics or N-containing aromatics).

The mass and UV spectra, as well as the SDF behavior on RP-HPLC at different
20 pH's, suggest that the active material is an aromatic charged composition of matter.

Example 3 - Biological Experimental Procedures

Continuous cell lines

Cell lines included the human myelomonocytic cells HL-60, GM-CSF-dependent human myelomonocytic cells LK (established in the inventors' laboratory) and the
25 murine monocytic cell line WEHI. Cell lines were maintained by sub-culturing every 3-4 days at 2.5×10^5 cell/ml in either alpha minimal essential or RPMI-1640 media (GIBCO, Grand Island, NY) supplemented with 10-20% fetal calf serum

-26-

(FCS) (Bio Labs Jerusalem, Israel). The cultures were incubated at 37°C, in a humidified atmosphere of 5% CO₂ in air. The concentration of viable cells was determined by the trypan blue exclusion technique. Total cell count was performed using a Coulter counter.

5 Cell proliferation assay

HL-60 or other cells, were cultured at $1.3 \times 10^5/\text{ml}$ in 24-well dish and medium containing 0.5% heat-inactivated FCS and various dilutions of the tested fractions. DNA synthesis was determined on day 4 by incorporation of ³[H]-thymidine (1mCi/150. μ l) (5 mCi/mmol, ICN Radiochemicals, Irvine, CA) added 12 hrs before
10 harvesting. One unit of SDF was defined as the amount per milliliter required for 50% decrease in ³H-thymidine incorporation.

Cell differentiation assays

Morphology: Cytospin (Shandon, Cheshire, UK) prepared slides were stained with May-Grunwald Giemsa. The percentage of macrophage-like cells was determined
15 by scoring at least 100 cells.

Phagocytosis tests

Polystyrene latex particles (3.2 μ diameter) (Sigma, St. Louis, MO) were added on day 3 of the assay as follows: Human serum was diluted 1:1 with saline and filtered through a 0.45 μ filter (to remove insoluble particles). Then, 5×10^7 beads were
20 added per ml diluted serum and incubated for 30 min at 37°C. This suspension was added at 0.1 ml/ml culture. Following 24 hr incubation, the cells were collected, washed twice with medium (in order to remove free latex particles) and the percentage of phagocytic cells was determined by scoring at least 200 cells, under an inverted microscope. An automatic phagocytosis test was performed by adding
25 fluorescent 2 μ latex particles (Polyscience, Warrington, PA) to the culture as described. Following 24 hrs incubation, a sample was directly analyzed in the Fluorescence Activated Cell Sorter (FACSTAR Plus, Becton-Dickinson).

Primary leukemic cells

Cells were obtained from patients admitted to the Hematology department of The Hadassah Medical Center, Ein Karem, Israel. Peripheral blood (PB) and bone marrow (BM) cells were collected in preservative-free heparin. Mononuclear-enriched fractions were prepared by Ficoll Hypaque (Pharmacia, Milan, Italy) density gradient centrifugation, washed and frozen in liquid nitrogen. Prior to each experiment, cells were thawed and cultured at 1×10^6 cells/ml in alpha medium supplemented with 20% FCS and 10% conditioned medium from bladder carcinoma cell cultures (5637-CM).

Leukemic cell cloning

300-1000 cells/ml were seeded in semi-solid medium composed of 0.83% methylcellulose (Fisher Scientific company, Fair Lawn, NJ) or 0.3% Bacto agar (Difco laboratories, Detroit, MI) in alpha medium supplemented with 10% FCS. Colony number was scored with an inverted microscope on day 8. In order to determine cellular maturation, single colonies were picked up with a fine capillary tube, smeared on a glass slide and stained.

Cloning of normal progenitors

Direct cloning: Peripheral bone mononuclear cells or BM cells obtained from normal volunteers were isolated by centrifugation on a gradient of Ficoll-Hypaque, and cloned in methylcellulose-containing alpha medium. For myeloid colonies, 30% FCS and CSF, in the form of 5637-CM or 100 U/ml GM-CSF, or IL-3 (Genetic institute, Cambridge, MA), 1% deionized BSA, 1×10^{-5} M 2-mercaptoethanol and 1.5 mM glutamine were added. For erythroid colonies, the culture included 30% FCS, 1% deionized BSA, 1.5 mM glutamine, 2-mercaptoethanol and 0.5-2 U/ml of erythropoietin (r-HuEPO, Cilag AG International, Zug Switzerland). One ml of the mixture containing either 2×10^5 BM or 5×10^5 PB cells was dispersed in 35 mm non-tissue culture dish (Falcon, Oxnard, CA). All semi-solid cultures were incubated at 37°C in a sealed incubator in humidified atmosphere of 6% O₂, 7% CO₂ and 87%

N₂. Colonies were scored with the aid of an inverted microscope. The cellular composition of colonies was confirmed by picking individual colonies, preparing a smear on a glass slide, and staining first with benzidine, and then, with Giemsa.

Indirect Cloning: Cells were prepared as described for direct cloning, were first incubated for several days in liquid culture supplemented with or without the indicated growth factors or tested fraction, then washed, and cloned in semi-solid medium as described above.

Example 4 - Effects of SDF or its complex with CP on different cell cultures

Effect on normal hemopoietic cells

10 *Expansion of normal hemopoietic progenitors* - In order to test the effect of SDF on early and late normal hemopoietic progenitors a two-phase culture procedure was employed:

Phase 1 (Expansion phase) - Light density BM or PB cells were incubated for several days in liquid medium supplemented with SDF.

15 *Phase 2* (Clonal phase) - At the end of phase 1, cells were harvested, washed and recultured in either semi solid medium or in liquid medium supplemented with either recombinant human late growth/differentiation factors like GM-CSF, Epo, or 5637-CM, which contains GM, G-CSF, IL-6, IL-1 and other cytokines, but not Epo. When BM or PB cells were incubated in Phase 1, for several days, with SDF as a
20 sole factor, and then cloned (Phase 2) in SDF-free semi-solid medium, a significant expansion in the number of CFU-C was obtained (Fig. 9 A-B). The lineage specific differentiation of the colonies in Phase 2 depended on the late factor present, e.g., Epo for CFU-E and GM-CSF for CFU-GM. When phase 1 (expansion phase) was omitted and the cells were cloned directly in semi-solid medium, SDF by itself did
25 not support colony development. When added to phase 2 with late factors, SDF had a small or no stimulatory effect (data not shown).

Like other early factors such as SCF, IL-1 and IL-6, SDF is mostly active in the expansion phase of pre CFU-C, and by itself cannot support the proliferation and

differentiation of CFU-C. It probably stimulates the proliferation of pre CFU-C, possibly by direct stimulation of expansion of CD34⁺ cells or by activation of accessory cells. Further proliferation and differentiation of CFU-C depends on the presence of late growth/ differentiation factors.

5 *Expansion of normal CD34⁺ cells* - Peripheral blood mononuclear cells were cultured in serum-containing liquid medium in the presence of SDF with no added cytokine. CD34⁺ cells were enumerated by flow cytometry at the initiation of the culture and at different days thereafter. The results demonstrate an average 10-fold increase in CD34⁺ following one week culture (Fig. 10).

10 *Expansion of normal dendritic cells* - As can be seen from Fig. 11, SDF induces commitment/expansion of PB CFU-dendritic. Using SDF in phase 1, up to 80% dendritic colonies in phase 2 were obtained from non-enriched PB mononuclear cells (Fig. 11).

Stimulation of hemopoietic progenitors from patients with pure red cell aplasia -
15 Erythroid progenitors from patients with pure red cell aplasia fail to develop both *in vivo* and *in vitro*. However, when treated with SDF in phase 1, PB samples from affected children developed into large and red (hemoglobin-containing) colonies in phase 2 (Fig. 12).

Effect on leukemic cells

20 *Acute myeloid leukemia* - SDF was found to be active, at very low concentrations, on different myeloid leukemic established cell-lines, e.g. HL-60 promyelocytic leukemic cells (Table 2), WEHI monoblast-like cells (Table 3), LK, a GM-CSF dependent myeloid leukemic cell line established in the inventors' laboratory (Table 4), as well as on freshly explanted cells from patients with various forms of myeloid
25 leukemia (Tables 5-6). In these, SDF-supplemented cultures, the leukemic population underwent differentiation into fully mature macrophages. The cells changed their shape into typical macrophage morphology (Table 2), acquired

macrophage functions such as mobility in semi-solid agar medium (assayed by the change of the colony morphology from "tight" to "diffused" (Table 3), the ability to phagocyte foreign particles (Tables 2 and 6), and to produce "non-specific" esterase (NSE) activity (Table 6).

Table 2

Effects of SDF on HL-60 cells

SDF dilution	Macrophage-like morphology	Phago-cytosis (%)	Inhibition of thymidine uptake (%)	Inhibition of colon formation (%)
1:100	++++	89	90	95
1:200	+++	78	85	90
1:400	+++	65	75	88
1:800	++	57	70	72
1:1600	+	45	63	69
1:3200	±	28	49	58
0	-	0		

*HL-60, at 1.3×10^5 cells/ml, were cultured in medium supplemented with 0.5% heat inactivated FCS. Phagocytosis, cell morphology and DNA synthesis were determined on day 4. Morphology score of the results are indicates as follows:-
 10 (+++++) represent 80-100 % macrophage-like cells, (++++) represent 60-80%, (++) represent 40-60%, (+) represent 15-40% and (±) represent 5-15% macrophage-like cells. For colonies, 600 cells were cloned per ml in a-MEM, 10% FCS and 0.3% Bacto agar and several dilutions of SDF.

For comparison, the effect of other growth and proliferation factors of the same cell
 15 lines was examined. As shown in the following table, SDF greatly inhibits colony formation.

-31-

Table 3

Effect of SDF and other growth and differentiation factors on

	Colonies			
	Tight	Partially diffused	Diffused	Total
<u>HL-60 cells</u>				
Control	301	11	1	313
G-CSF(1000U/ml)	325	12	0	337
IL-1(100U/ml)	332	8	1	341
G-CSF + IL-1	338	14	0	352
Retinoic acid	0	49	114	163
SDF dilution: 1:200	0	2	5	7
1:400	0	10	2	12
1:800	2	17	16	35
1:1600	5	28	22	55
<u>WEHI cells</u>				
Control	78	20	17	115
G-CSF (100 U/ml)	25	55	51	132
IL-1 (100 U/ml)	0	16	94	11
G + IL-1	2	40	89	131
SDF dilution: 1:100	0	63	11	74
1:200	2	77	20	98
1:400	5	80	26	111
1:800	21	62	11	99
1:1600	53	42	15	109
1:3200	43	32	12	87

*HL-60 (600 cells/ml) and WEHI (300 cells/ml) were cloned as with described for

Table 2. Cells in α -medium, 10% FCS and 0.3% Difco Bacto agar were plated with

5 different dilutions of SDF, IL-1, G-CSF and retinoic acid. Colonies were classified

according to their morphology as tight (undifferentiated), partially diffused (partially differentiated) and diffused (fully differentiated). The number of colonies was determined after 8 days, as the mean of the results obtained in two independent experiments.

5

Table 4

The effect of SDF on the proliferation of LK cells

SDF dilution	Without GM-CSF		With GM-CSF	
	CPM	Growth inhibition (%)	CPM	Growth inhibition (%)
Control	4778	0	17247	0
1:100	1552	68	4700	73
1:200	2003	59	4980	72
1:400	2017	59	5081	71
1:800	1719	64	5739	67

*LK cells (a GM-CSF dependent myeloid leukemic cell line) were cultured at 5×10^4 cells/ml in α -MEM and 10% FCS. The cultures were supplemented with or without GM-CSF (100 U/ml) and different dilutions of SDF. DNA synthesis was determined on day 4 by incorporation of ^3H -thymidine. The results are expressed as CPM/ml culture and percent of growth inhibition.

10

It is clear from the above that SDF is capable of inhibiting the growth of myeloid leukemia cells also in the absence of GM-CSF.

Table 5 (A)-(D)**The effect of SDF on freshly explanted AML cells****A: Patient 1 - M1/M2 cells**

SDF dilution	SDF		SDF + GM-CSF	
	CPM	Inhibition (%)	CPM	Inhibition (%)
Control	7000	0	11000	0
1:100	1540	78	1640	85
1:200	1435	79	2438	78
1:400	2296	67	2107	81
1:800	2892	59	3108	72
1:1600	4300	39	4834	57
1:13200	5971	15	5894	46

B: Patient 2 - M1 cells

SDF dilution	CPM		Inhibition (%)		Phagocytic cells (%)	
	(-) CSF	(+) CSF	(-) CSF	(+)CSF	(-) CSF	(+)CSF
Control	6000	12000	0	0	49	63
1:200	900	626	85	95	96	93
1:400	800	588	87	95	95	95
1:800	872	N.D.	86	N.D.	90	N.D.
1:1600	1000	N.D.	83	N.D.	85	N.D.

C: Patient 3 - AML M4 cells (Acute Monocytic Leukemia)

Culture conditions	CPM	Inhibition (%)	Phagocytic cells (%)	
			day 4	day 7
FCS 10%	9840		12	26
FCS 10% + SDF 1:200	2767	72	45	70
AML serum 10%	14120		5	10
AML serum 10% + SDF 1:200	3800	73	15	50

*Primary AML cells were cultured at 1×10^6 cells/ml in a-MEM and 10% FCS or autologous serum with or without SDF (1:200 dilution). The cultures were labeled with ^3H -thymidine after six days.

5 D: Patient 4 - Mixed AML-ALL cells

Culture conditions		Sudan Black (%)	NSE* (%)	Phago-cells (%)
Prior to culture		20	0	1
After 6 days in culture	FCS (10%)	7	7	10
	FCS (10%)+SDF (1:200)	8	37	50
	Autologous serum (10%)	46	6	5
	Autologous serum (10%)+SDF (1:200)	16	34	45

Primary mixed AML-ALL cells were cultured at 1×10^6 cells/ml as indicated. Cells were analyzed on the sixth day of culture.

In all four experiments, (A) to (D), non specific esterase and phagocytic cells were determined on the sixth day.

Upon exposure to SDF, as a result of terminal cell differentiation, the leukemic cells lost their ability for proliferation and self-renewal, as determined by thymidine uptake and cloning in semi-solid medium (Table 2 and Fig. 13).

Although full maturation occurred after 3-4 days, exposure to SDF for one day sufficed to induce irreversible commitment to terminal differentiation (Table 6), including the loss of self-renewal capacity.

Table 6

The effect of SDF on self renewal and commitment
to differentiation of HL-60 cells

Incubation with SDF (days)	No. of colonies per plate	Phagocytic cells (%)
0	150	3
1	1	18
2	1	65
3	0	74

*HL-60 cells were incubated with SDF (1:200 dilution). After 1, 2 or 3 days, the cells were washed and 1×10^3 viable cells were plated in semi-solid culture. Remaining cells were replanted in suspension. Colony number was determined on day 7. Phagocytic capacity was determined on the sixth day.

Leukemic cells, such as HL-60 cells, inhibit the development of normal BM cells. However, when HL-60 cells were treated for 24 hrs with SDF, they lost their ability to inhibit normal hemopoietic development (Table 7).

Table 7

The effect of HL-60 cells on development of normal BM colonies

	% of control (No. of BM colonies)
Control	100% (320)
Untreated HL-60 cells	8% (26)
SDF-treated HL-60 cells	56% (180)

*Viable HL-60 cells (1×10^5) were incubated for 24 hours with or without SDF (1:200 dilution) and then plated in agar as an underlay of normal BM cells. The number of BM colonies was determined on day 12.

Chronic myeloid leukemia (CML)

The effect of SDF on the proliferation ability of cells derived from chronic myeloid leukemic patients (CML) was also examined by the inventors (Table 7). SDF was cultured with CML colony forming cells (CFU-C) in a direct cloning procedure, utilizing a semi-solid culture and in an indirect cloning Procedure (two phase culture), as described herein before. These Cells retained their ability to differentiate but showed abnormal proliferation pattern. Therefore, it was concluded that SDF is capable of inhibiting the potential of these progenitors to proliferate and to develop colonies. When CML cells were grown in liquid cultures, SDF had no apparent toxicity. These cultures contained mainly myeloid precursors at various stages of maturation.

These results suggest that SDF inhibits early (pre-CFU-C) CML progenitors (either by direct inhibition of their proliferation or indirectly by inducing rapid differentiation), but has no deleterious effect on the more mature cells.

-37-

Table 8**SDF effect on CML Colony Forming Cells (CFU-C) Inhibition (%)**

Fraction dilution	Direct cloning	Indirect cloning
1:100	100	80
1:200	100	60
1:500	70*	20
1:1000	40*	0

*Small colonies compared to control. Number of colonies was determined on day 14 and the results are expressed as percent of inhibition compared with the control.

- 5 As noted above, The effect of SDF was assayed on CML colony forming cells by the direct and indirect colony assays. For direct cloning, 0.5×10^6 cells/ml were cultured in semi solid medium supplemented with GM-CSF and Epo, with or without the indicated dilution of SDF. For indirect cloning, 0.5×10^6 cells/ml were cultured first in liquid medium with the indicated dilution's of SDF. After 3 days,
- 10 non adherent cells were recovered, washed, and assayed for CFU-C in semi solid medium supplemented only with GM-CSF and Epo.

Effect of SDF on normal hemopoietic progenitors

- Different growth factors e.g. G-CSF, GM-CSF are currently used in bone marrow transplantation. They have been shown to shorten neutrophil recovery time after
- 15 transplantation (and chemotherapy) by stimulating myeloid progenitors. However, since myeloid leukemic cells have receptors for these factors, the proliferation of residual malignant cells is also stimulated. The following reveals that SDF by itself or in combination with GM-CSF potentiates the proliferation of normal progenitors, but inhibits "spontaneous" and GM-CSF stimulated proliferation of
- 20 myeloid leukemic cells, thus having a dual effect: eradication of leukemic cells concomitantly with stimulation of the normal ones.

Example 5**Effect of SDF on angiogenesis**

The inventors have found that highly purified plasma derived SDF contains a potent inhibitory activity on endothelial cell proliferation, in vitro (Table 8).

Table 9**Effect of SDF on proliferation of endothelial cells**

Fraction		CPM
control		35000
S-Sepharose derived fraction		
dilution	1:1000	15000
	1:500	9000
	1:250	5000
SDS gel derived fraction		
dilution	1:100	3500
	1:50	1200
	1:25	550

*Endothelial cells derived from bovine aorta were seeded in 1 ml aliquots per well (5000 cells/ml) into 24-well cluster dishes. The culture medium was supplemented with 10% heat inactivated horse serum. After 5 hr. incubation, 40 μ l of buffer or buffer supplemented with various concentration of the SDF-containing fractions were added to each well. Following 4 days of incubation, the cultures were pulsed labeled with 3 H-thymidine for 20 hrs and then harvested. The results are expressed as counts per minute (CPM).

Example 6**Effect of PQQ on hemopoietic progenitor cells**

The inventors have found that PQQ and related compounds induce differentiation in myeloid leukemic cells and stimulate the growth of early hemopoietic progenitor cells. The described effect is exemplified in Table 10.

Table 10**The effect of PQQ on the proliferation of HL60 cells**

Concentration ($\mu\text{g/ml}$)	Growth inhibition (%)
5	60
1	40
0.5	40
0.1	30
0.05	30
0.01	0

Further, the effect of PQQ on normal hemopoietic progenitor cells in an indirect cloning system was examined (Table 11).

Table 11**Effect of PQQ in an indirect cloning system**

Concentration ($\mu\text{g/ml}$)	Colony No.
5	148
1	130
0.1	109
0.01	89
0	70

CLAIMS

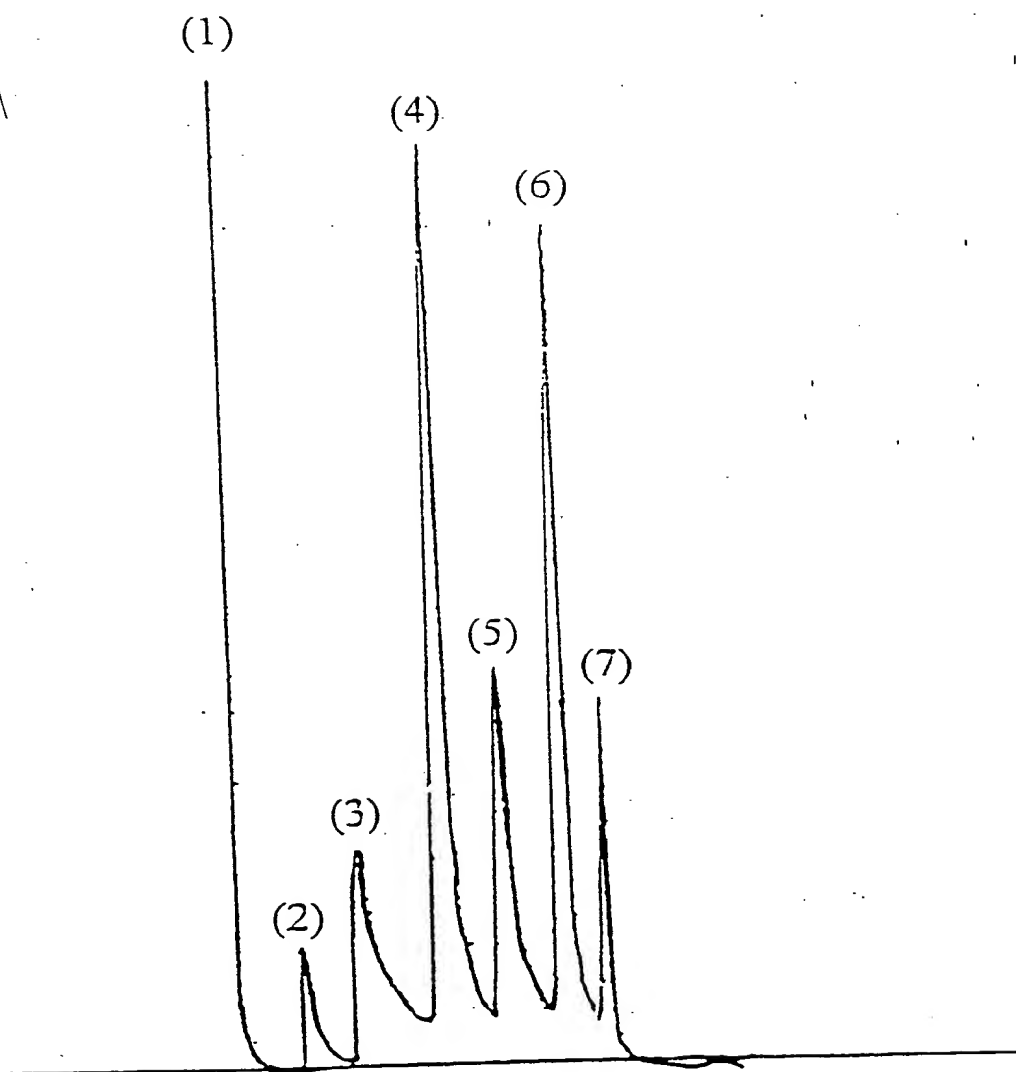
1. A biologically active serum-derived composition of matter (SDF), having a low molecular weight, being electrically charged at acidic pH and having absorption at 280 nm.
2. The SDF of claim 1 having a molecular weight of 316, as determined by electron spray mass spectrometry.
3. A method for the isolation and purification from plasma, a low molecular weight composition of matter, comprising the steps of:-
 - a) transferring plasma through an affinity column to obtain an electrophoretically homogeneous fraction, to give serum-derived factor in complex with ceruloplasmin (SDF-CP complex) which may optionally be further concentrated by transferring through an anion exchange column;
 - b) isolating SDF from its complex with CP obtained in step (a) by
 - (i) transferring the complex obtained in step (a) through RP-HPLC resource column employing elution buffer A, consisting of 0.05-0.01% trifluoroacetic acid (TFA) in water, and elution buffer B, consisting of acetonitrile, and collecting and combining two fractions, the first of said fractions being eluted at acetonitrile concentration of 0-2% and the second of said fractions being eluted at acetonitrile concentration of 13-17%; or
 - ii) extracting the fraction obtained in step (a) with an acidified solvent, wherein the active fraction is recovered from the organic phase;
 - c) purifying the material obtained in step (b) by a two-step RP-HPLC chromatography separation using a C18 column,
 - wherein in the first separation step, elution buffer A, consisting of 0.1% trifluoroacetic acid (TFA) in water (pH 2.5), and elution buffer B, consisting of 0.1% TFA in acetonitrile, are employed, and the fraction eluted at acetonitrile concentration of 0-2% is collected; and

-41-

- wherein in the second separation step, elution buffer A, consisting of 0.1% triethylamine in water, adjusted to pH 7.0, and elution buffer B, consisting of acetonitrile, are employed and the fraction eluted at acetonitrile concentration of 9-11% is collected.
4. The method of claim 3 in which said plasma is human plasma.
 5. The method of claim 4 in which said affinity column is a tentacle-agarose gel, derived using a reaction of Sepharose CL-6B or Sepharose 4B with chloroethylamine.
 6. The method of claim 4 in which said anion exchange chromatography is preferably DEAE or QAE Sephadex chromatography.
 7. SDF whenever obtained by the method of any one of claims 3 to 6.
 8. A biologically active complex comprising ceruloplasmin and the biologically active composition of matter (SDF) of claim 1.
 9. SDF according to claim 1 or its complex with ceruloplasmin according to claim 8, capable of inducing terminal cell differentiation of leukemic cells.
 10. SDF according to claim 1 or its complex according to claim 9 wherein said leukemic cells lose their ability to proliferate and their ability for self cell renewal.
 11. SDF according to claim 1 or its complex according to claim 9 capable of inducing loss of the ability of leukemic cells to proliferate and self cell renewal.
 12. SDF according to claim 1 or its complex with ceruloplasmin according to claim 8 capable of stimulating the proliferation of early, normal progenitor cells.
 13. SDF according to claim 1 or its complex according to claim 9 capable of inhibiting enhanced angiogenesis.
 14. SDF according to claim 1 or its complex according to claim 9 capable of *ex vivo* expanding normal stem and progenitor cells.
 15. Pharmaceutical composition for inhibiting enhanced angiogenesis comprising as active ingredient the SDF of any one of claims 1, 2 or 7, or its complex

- according to claim 8, and optionally further comprising pharmaceutically acceptable additives.
16. Pharmaceutical composition for inducing remission of tumors comprising as active ingredient the SDF of any one of claims 1, 2 or 7, or its complex according to claim 8, and optionally further comprising pharmaceutically acceptable additives.
 17. Pharmaceutical composition for maintaining tumor remission state comprising as active ingredient the SDF of any one of claims 1, 2 or 7, and optionally further comprising pharmaceutically acceptable additives.
 18. Pharmaceutical composition for expanding hematopoietic normal stem and progenitor bone marrow transplants comprising as active ingredient the SDF of any one of claims 1, 2 or 7, or its complex according to claim 8, and optionally further comprising pharmaceutically acceptable additives.
 19. Use of SDF of claims 1, 2, and 7 or its complex according to claim 8 in the preparation of a pharmaceutical composition for expanding hematopoietic normal stem and progenitor bone marrow transplants.

1/15

**Figure 1**

2/15

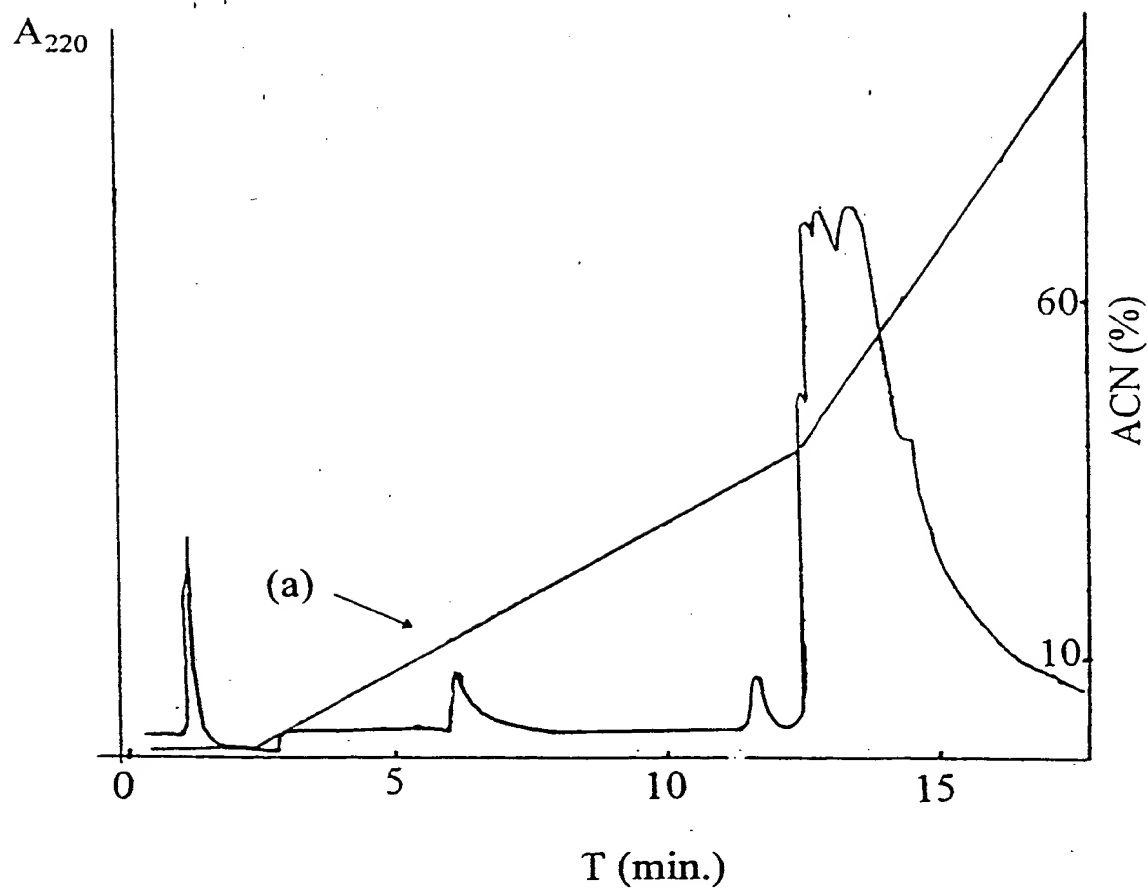
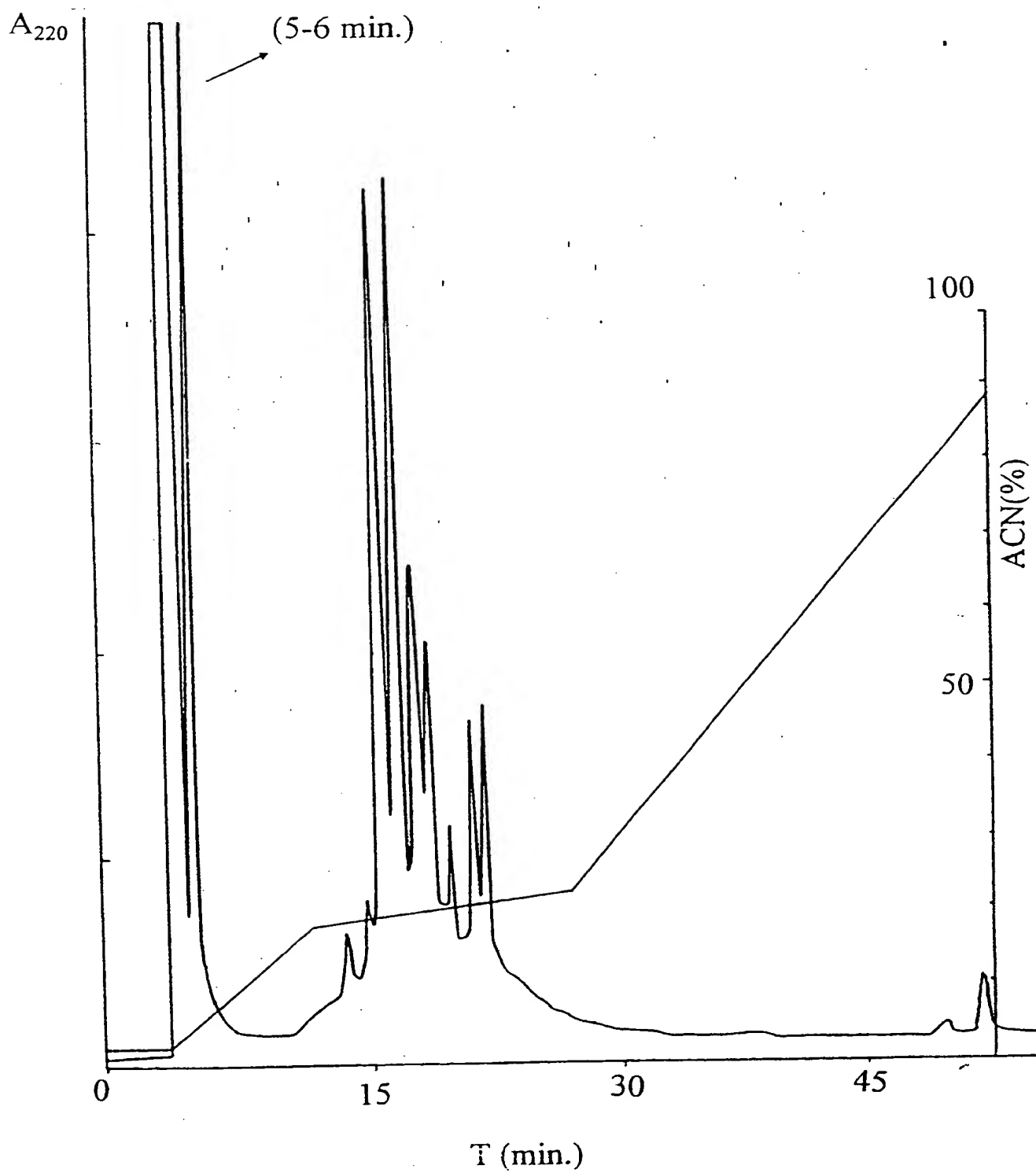


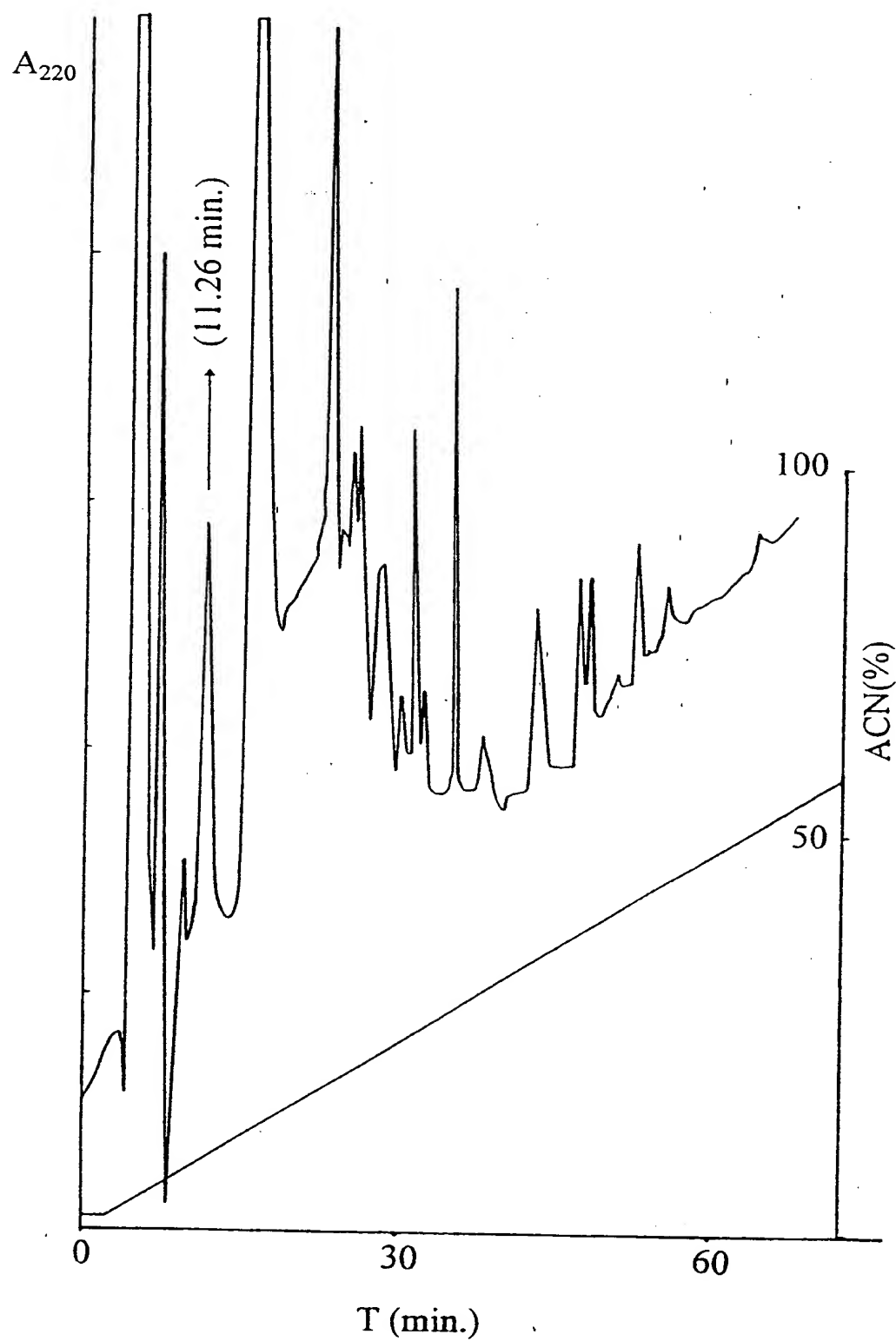
Figure 2

3/15

**Figure 3**

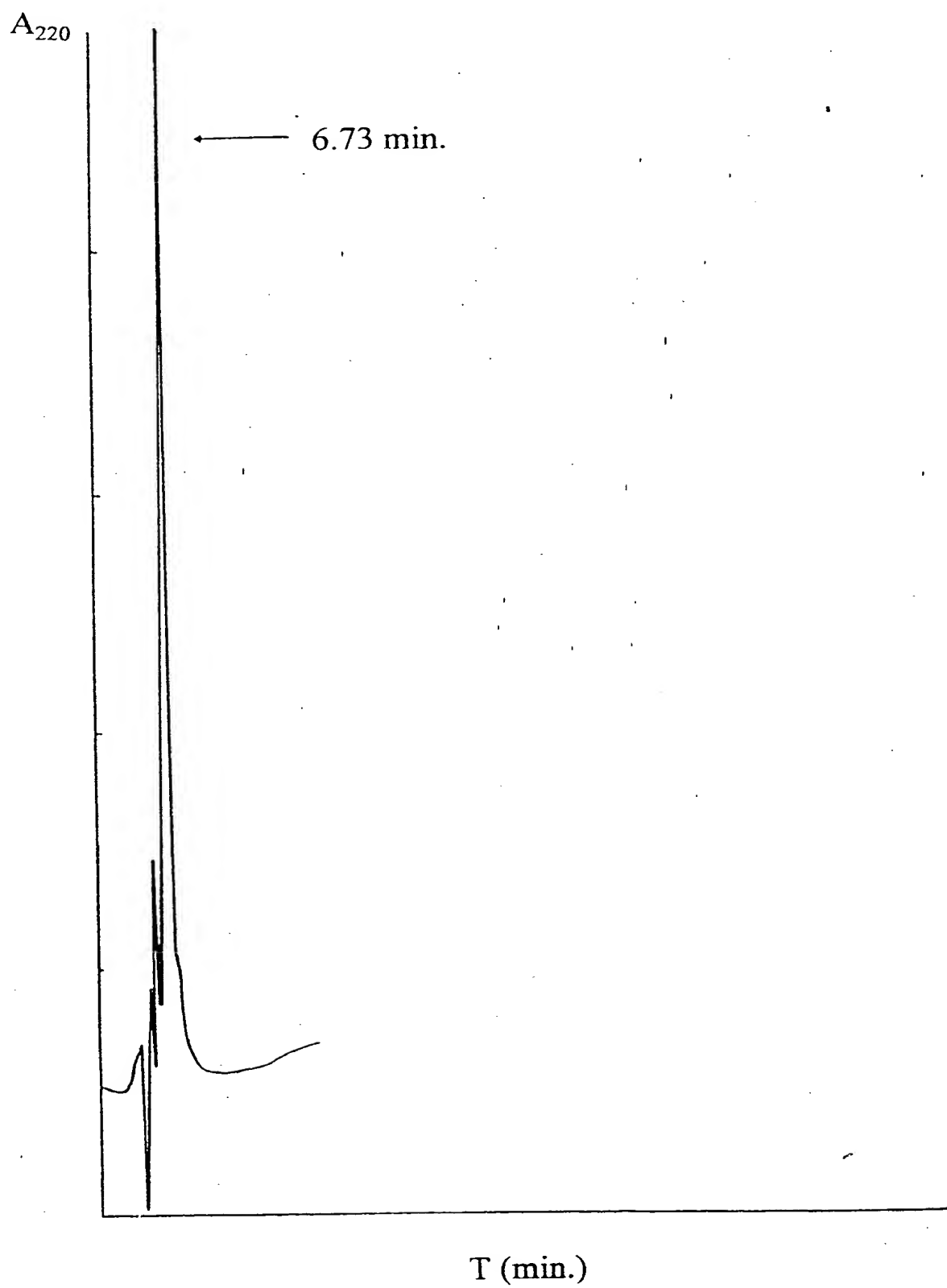
SUBSTITUTE SHEET (RULE 26)

4/15

**Figure 4**

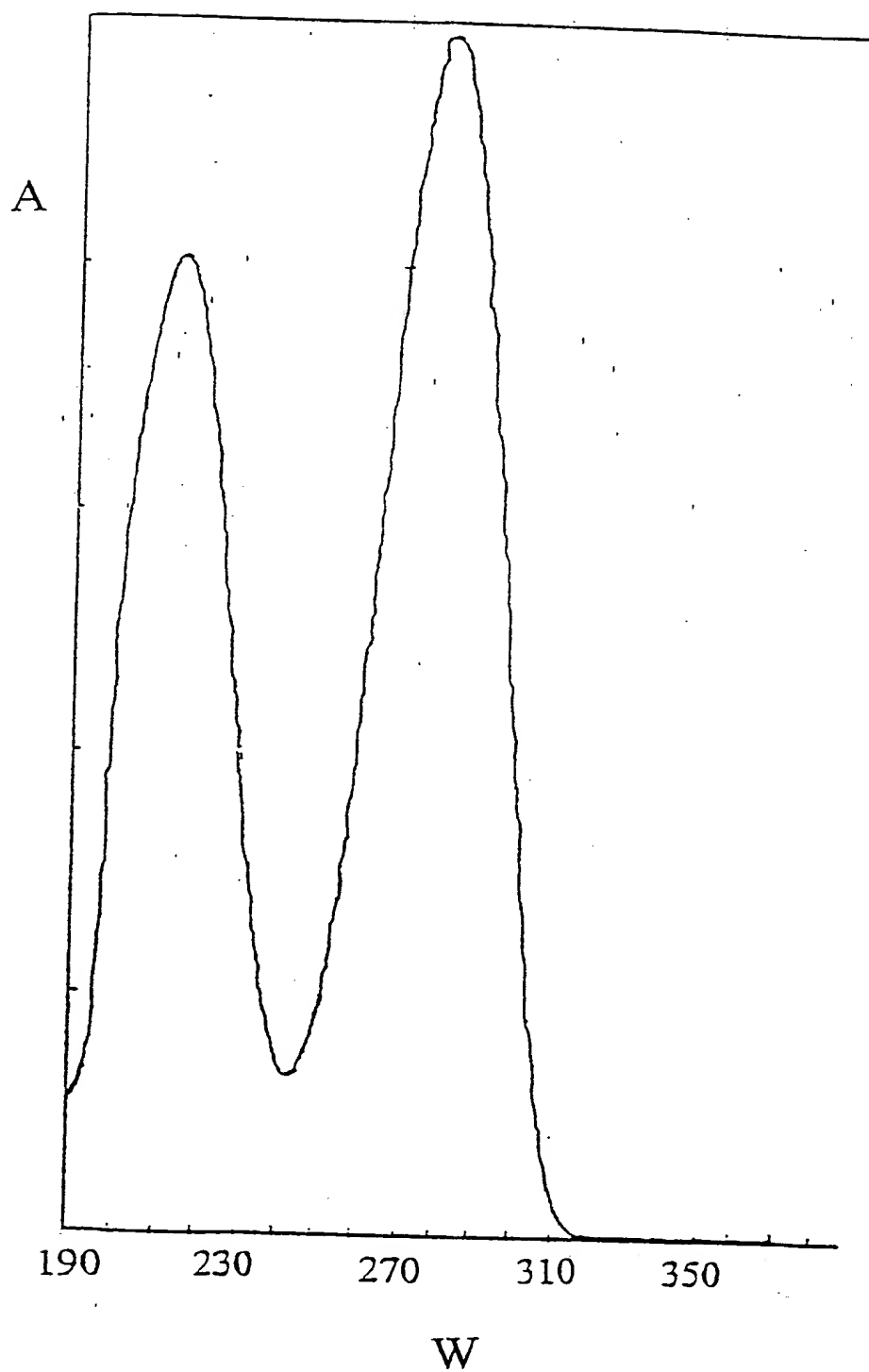
SUBSTITUTE SHEET (RULE 26)

5/15

**Figure 5**

SUBSTITUTE SHEET (RULE 26)

6/15

**Figure 6**

7/15

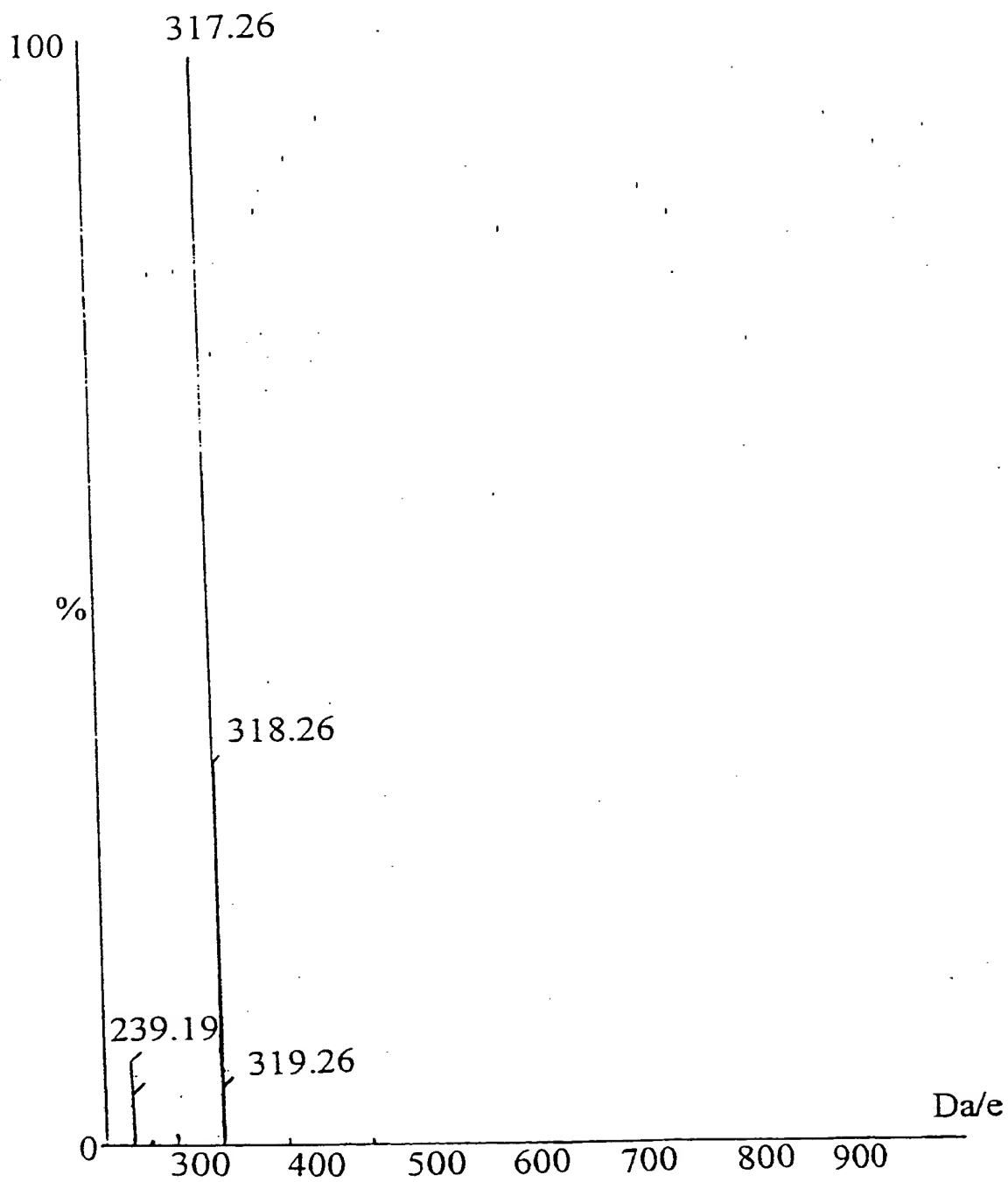


Figure 7

8/15

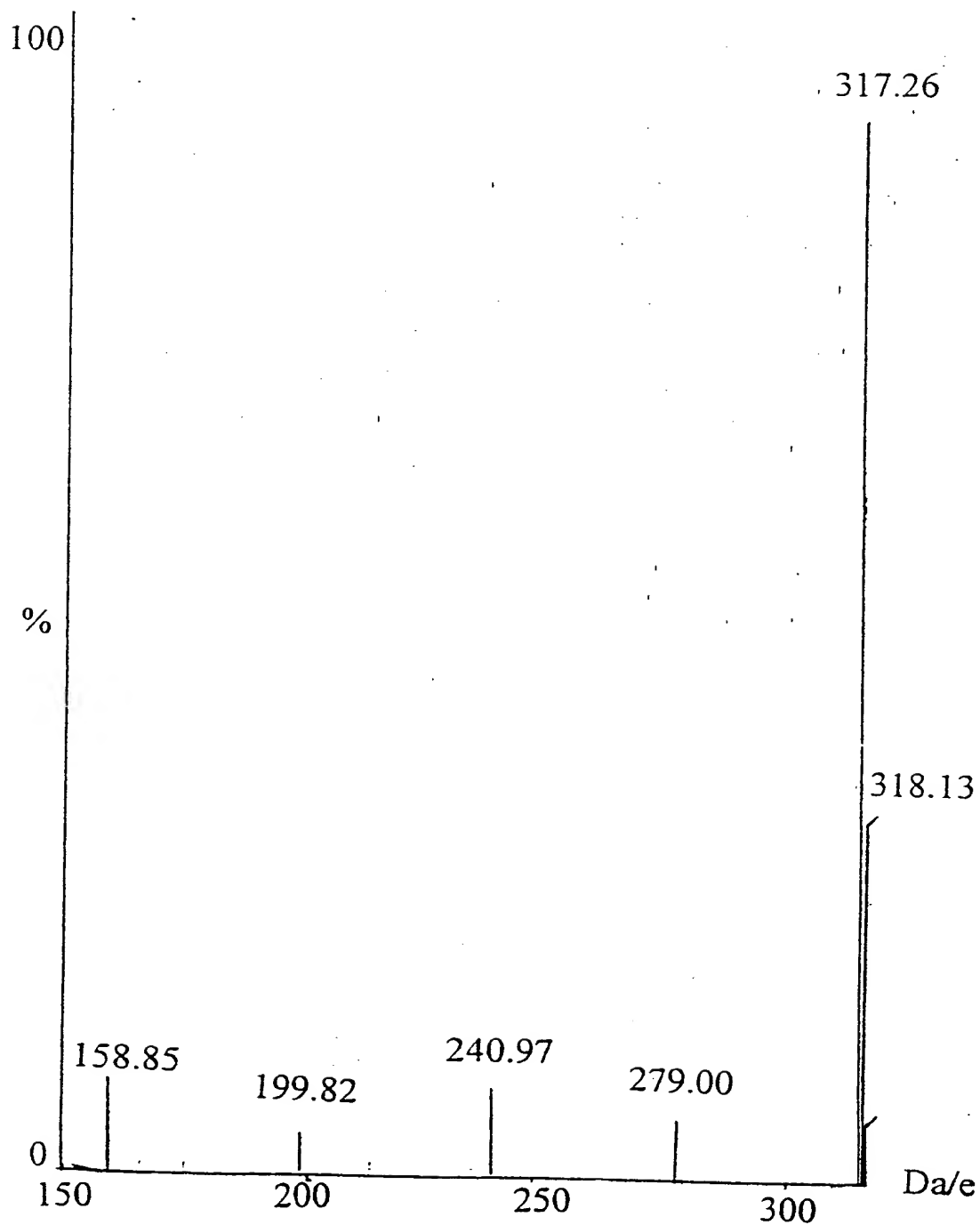
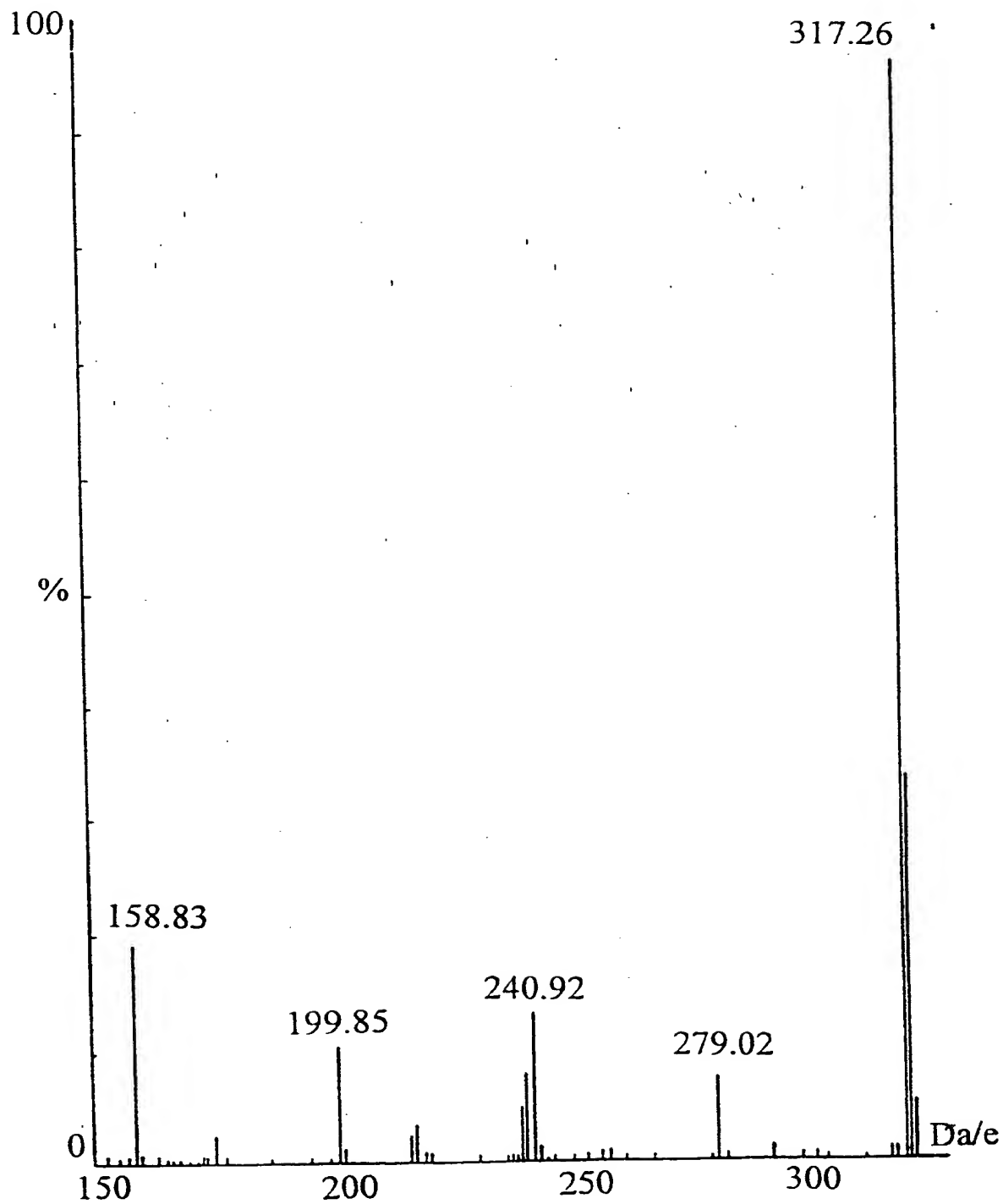


Figure 8(A)

9/15

**Figure 8(B)**

SUBSTITUTE SHEET (RULE 26)

10/15

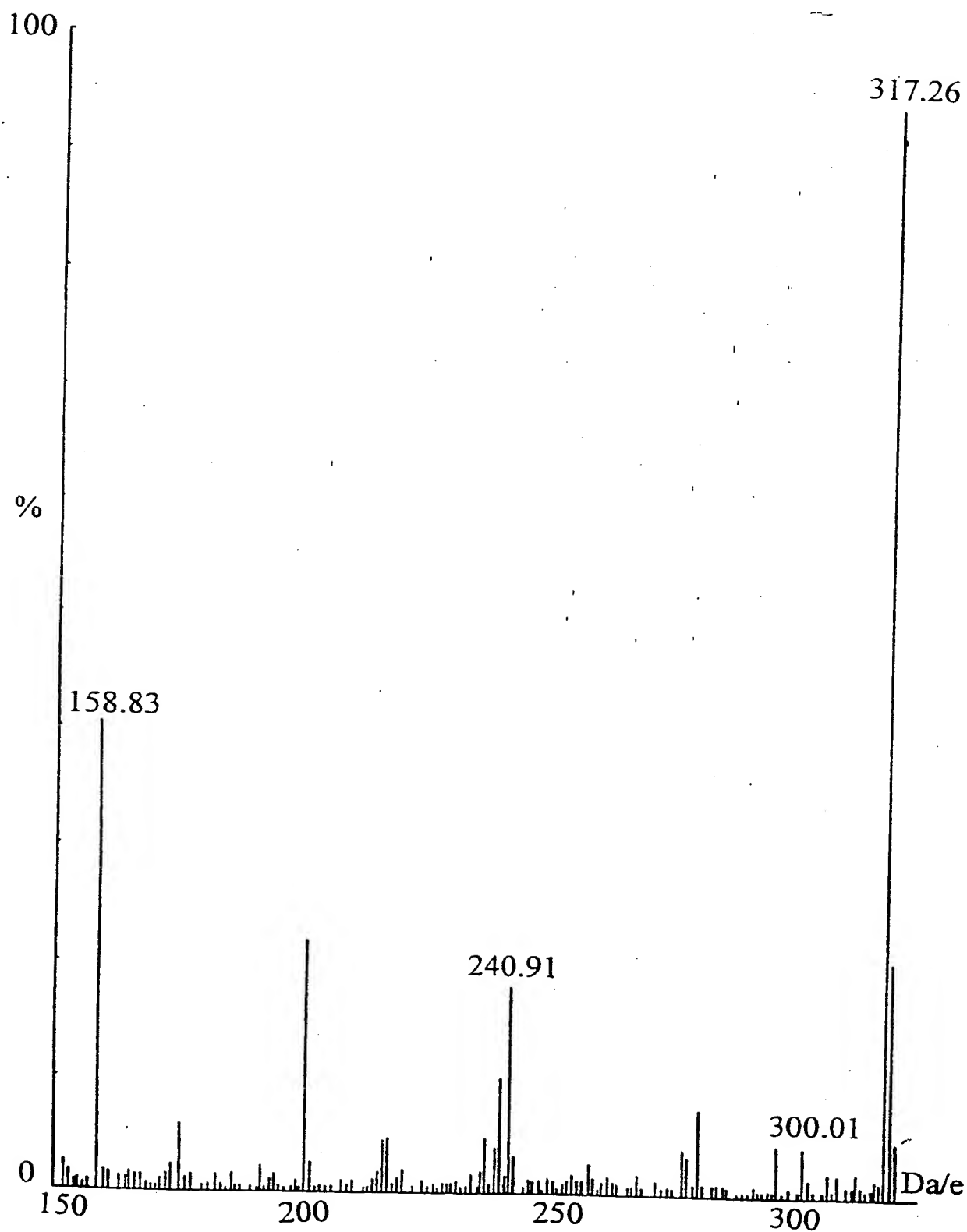


Figure 8(C)

SUBSTITUTE SHEET (RULE 26)

11/15

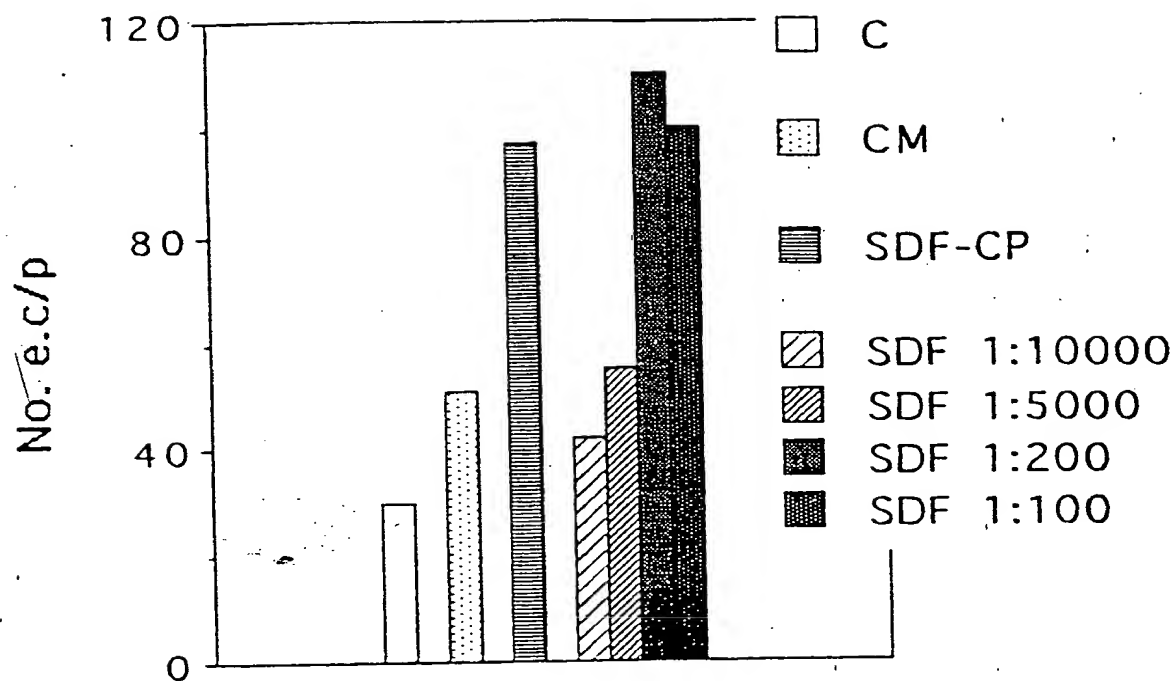


Figure 9(A)

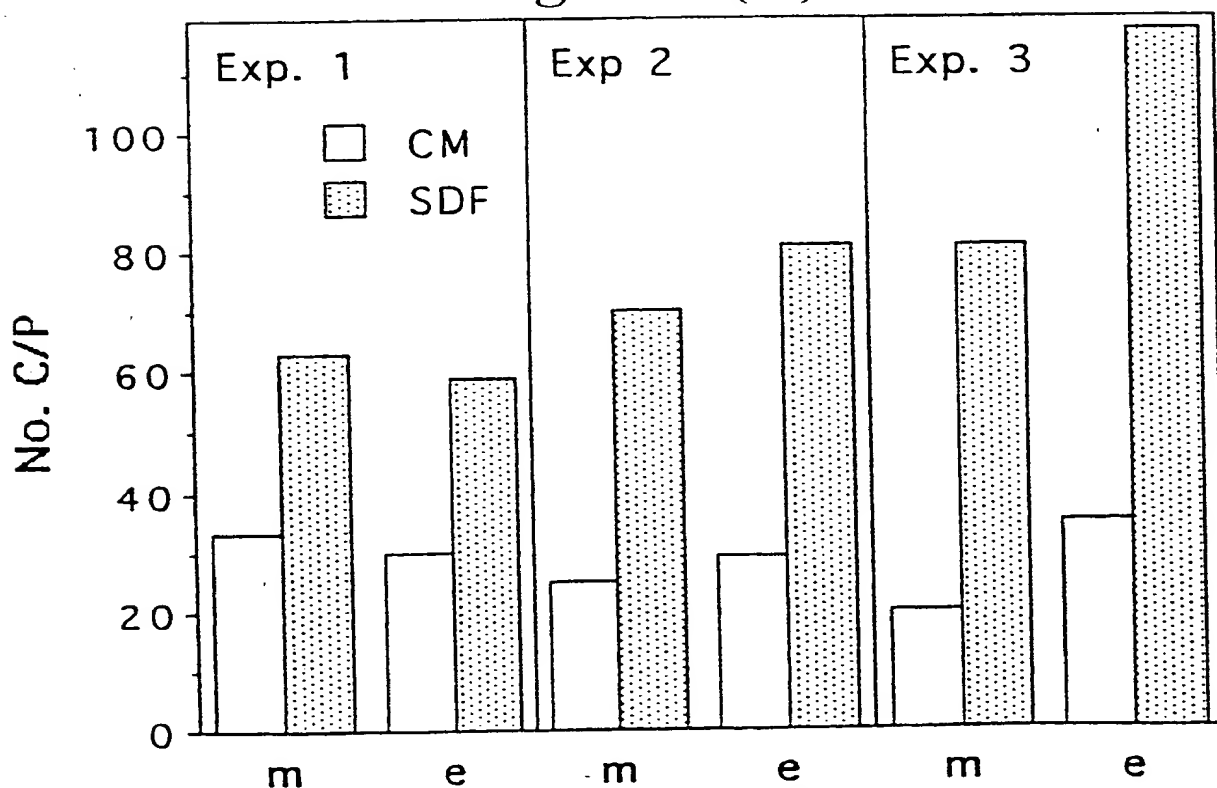
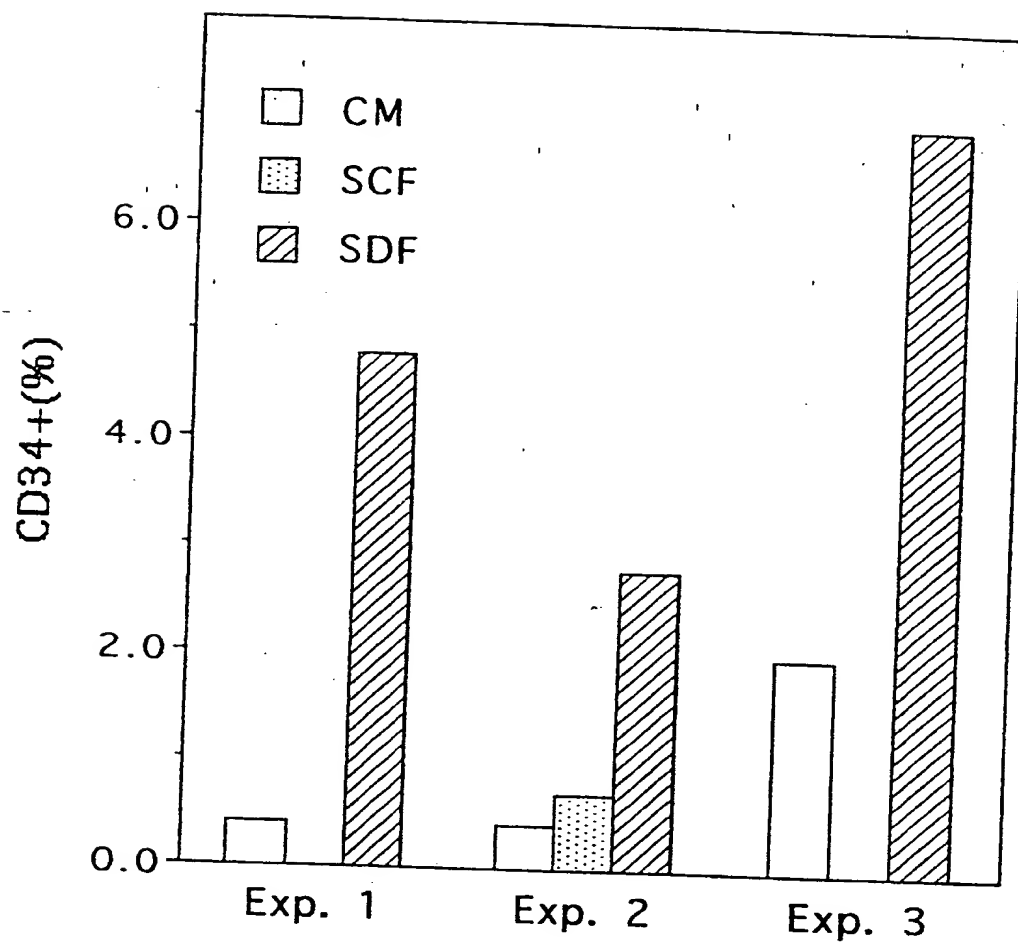


Figure 9(B)

12/15

**Figure 10**

13/15

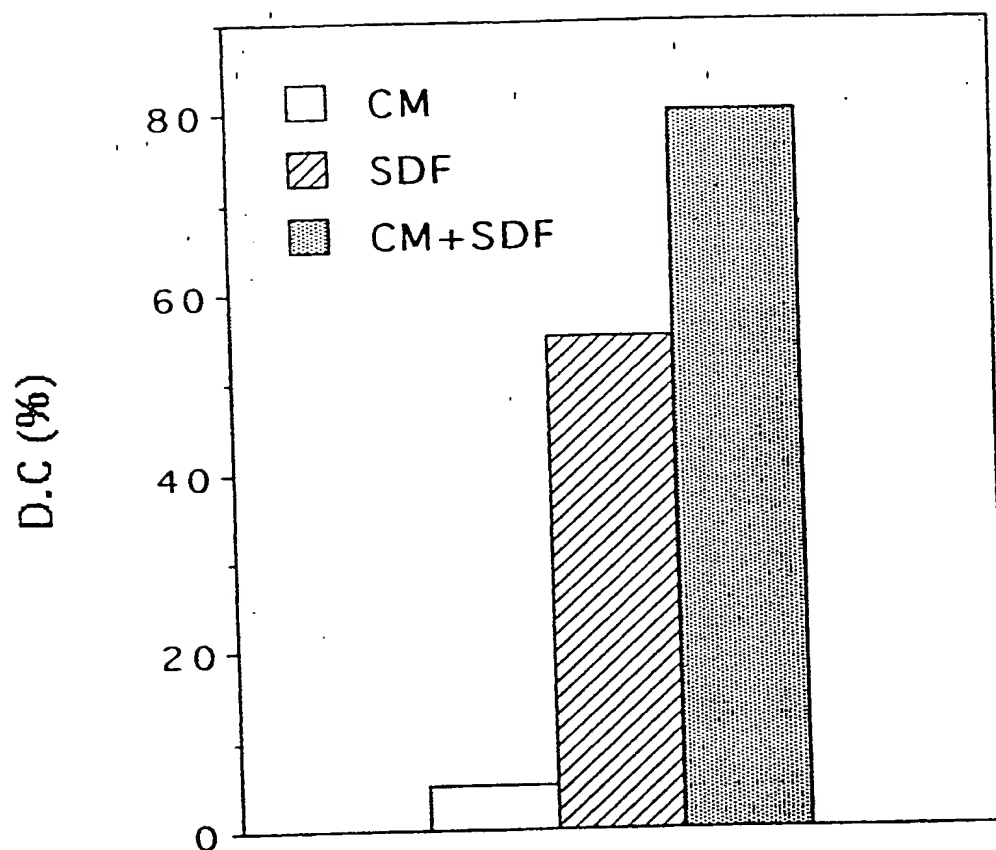


Figure 11

14/15

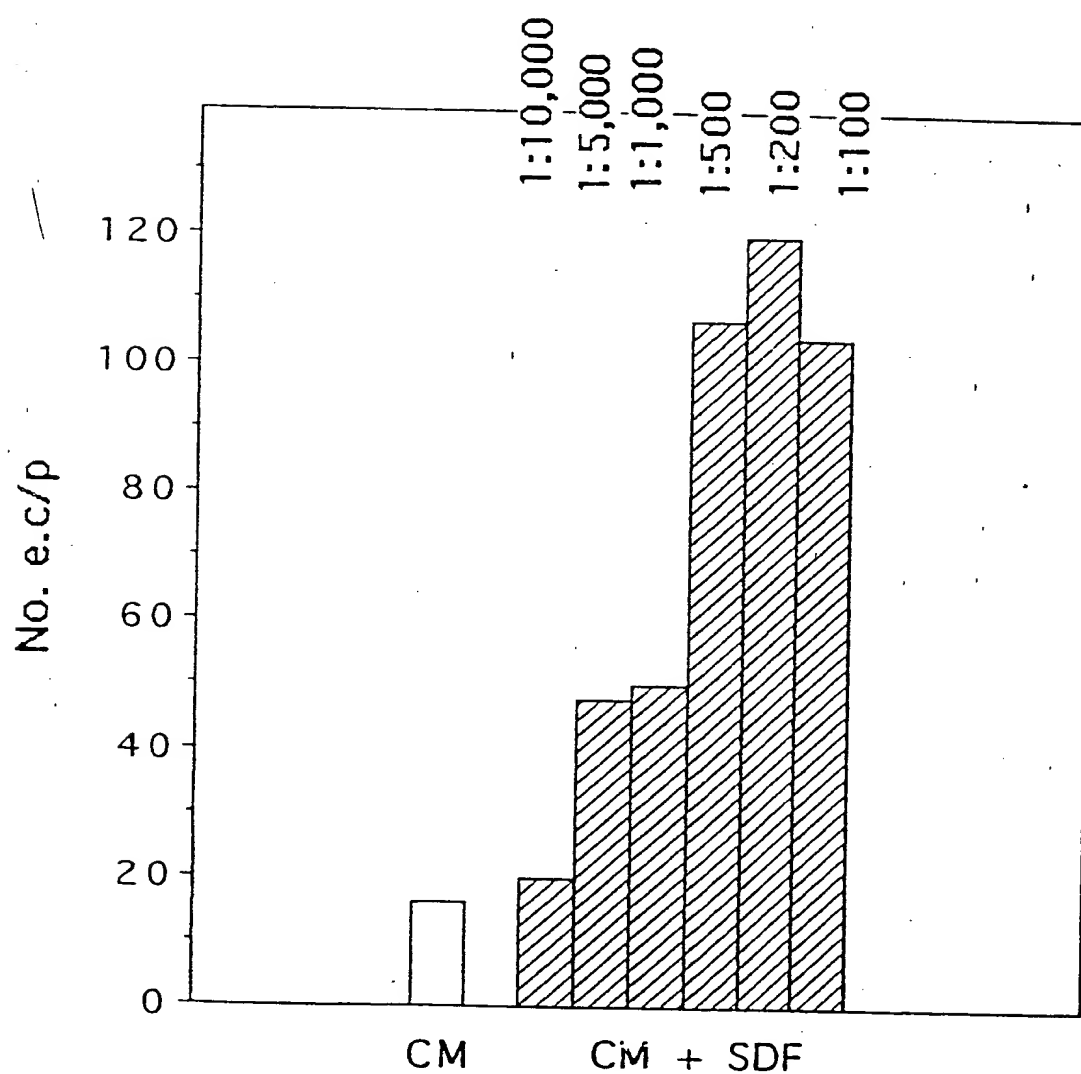


Figure 12

15/15

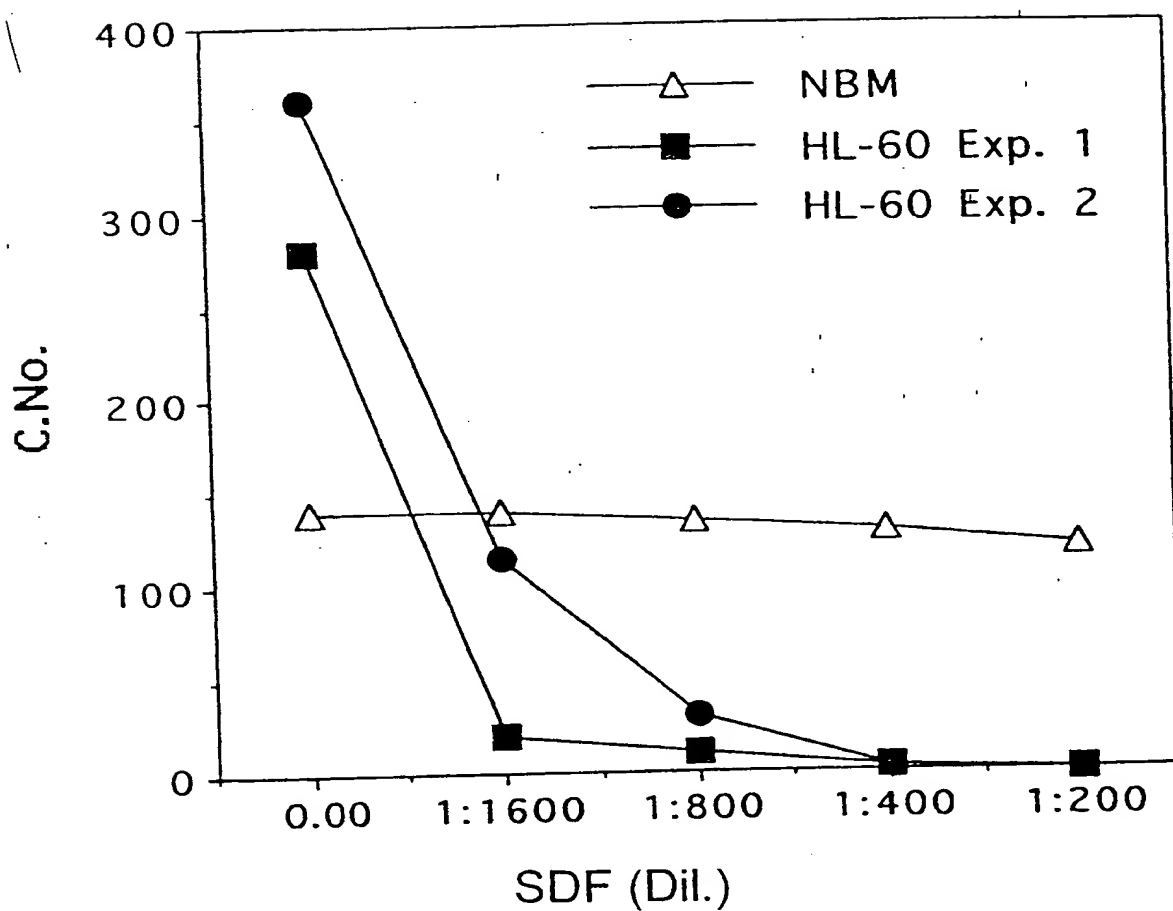


Figure 13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL97/00395

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 35/14, 35/16; C12N 5/00, 5/02, 5/06

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/325, 372, 372.1, 372.2, 373.3, 377, 405, 408; 514/509; 530/392

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DARWISH, H.M. et al. Mobilization of Copper(II) from Plasma Components and Mechanism of Hepatic Copper Transport. Am. J. Am. Physiol. Soc. 1984, Vol. 246, No. 9, pages G72-G79, especially, page G77, first paragraph under "Discussion".	1
Y	LEHNINGER, A.L. The molecular basis of cell structure and function. In: Biochemistry, 2nd Edition, Worth Publishers, Inc. 1975, pages 73-75, especially page 75, last entry in Figure 4-4.	1
X	GB 1304697 A (LAROCHÉ) 24 January 1973, see entire document.	8-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 MARCH 1998

Date of mailing of the international search report

30 APR 1998

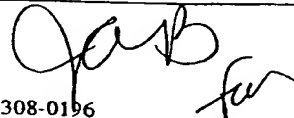
Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JANET M. KERR

Telephone No. (703) 308-0196



Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL97/00395

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STEINKUHLER, C. et al. Increase of Cu, Zn-Superoxide Dismutase Activity during Differentiation of Human K562 Cells Involves Activation by Copper of a Constantly Expressed Copper-deficient Protein. J. Biol. Chem. 25 December 1991, Vol. 266, No. 36, pages 24580-24587, see entire document.	8-14
X	JP 60-149529 A (SHIMIZU) 07 August 1985, see English translation of abstract.	8-14

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL97/00395

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 15-19
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL97/00395

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/325, 372, 372.1, 372.2, 373.3, 377, 405, 408; 514/509; 530/392

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, EMBASE, BIOSIS, WPIDS, CAPLUS

search terms: serum-derived factor, sdf, ceruloplasmin, leukocyte#, leukem?, lymphocyte#, stem, progenitor, low molecular weight, 316, complex?, serum, plasma, blood, copper-dihistidine, cu2his, adsorb?, aromatic, ion exchange, alpha-2-globulin, chromatograph?, reverse phase, tentacle-agarose, bone marrow, proliferat?, differentiat?, angiogen?

